Marcelo Dias Catarino

PHENOLIC CHARACTERIZATION AND EVALUATION OF THE ANTI-OXIDANT AND ANTI-INFLAMMATORY PROPERTIES OF ERIOCEPH-ALUS AFRICANUS AND GERANIUM ROBERTIANUM EXTRACTS

Dissertação de Mestrado em Biotecnologia Farmacêutica, orientada pela Doutora Susana Maria de Almeida Cardoso (Universidade de Aveiro) e pela Prof. Doutora Teresa Rosete (Universidade de Coimbra) e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

September 2015

UNIVERSIDADE DE COIMBRA

Marcelo Dias Catarino

Phenolic characterization and evaluation of the antioxi-dant and anti-inflammatory properties of *Eriocephalus africanus* and *Geranium robertianum* extracts

Dissertação de Mestrado em Biotecnologia Farmacêutica, orientada pela Doutora Susana Maria de Almeida Cardoso (Universidade de Aveiro) e pela Prof. Doutora Teresa Rosete (Universidade de Coimbra) e apresentada à Faculdade de Farmácia da Universidade de Coimbra

2015

“A person who never made a mistake

never tried anything new.”

Albert Einstein

AGRADECIMENTOS

Tendo chegado o final de mais uma etapa da minha vida, a gratidão que tenho para com todos os que direta ou indiretamente contribuíram para que eu pudesse alcançar esta meta é de uma tal dimensão que torna própria palavra "obrigado" numa forma ingrata de expressar todo o meu agradecimento.

Assim, sem mais demoras, tenho que agradecer em primeiro lugar à Doutora Susana M. Cardoso não só pela disponibilidade, apoio e orientação que me prestou durante esta jornada mas também por todos os ensinamentos, conselhos, motivação e boa disposição; por todas as chamadas de atenção, pelas oportunidades, amizade e momentos de descontração; mas sobretudo pela confiança depositada e por ter apostado em mim quando eu era ainda um passarinho no ninho à procura do meu primeiro estágio de licenciatura. Um sincero obrigado por me ter ajudado a crescer ao longo desta relação que já conta com alguns anitos, e por me mostrar como a ciência pode ter tanto de frustrante como emocionante.

Quero ainda estender os meus agradecimentos à Doutora Maria Teresa Cruz, também ela minha orientadora, por me ter acolhido junto da sua equipa e disponibilizado condições para realizar parte deste trabalho, pela transmissão de novos conhecimentos e também pela orientação, disponibilidade, simpatia e atenção.

Aos meus colegas do laboratório do IBILI, nomeadamente ao João Martins, Bruno Neves, Ana Rufino, Ana Silva, João Couto, Joana Liberal, Mónica Zuzarte, Margarida Neves, Cátia Sousa e Isabel Ferreira, quero agradecer também pela facilidade com que me integraram neste grupo, pelo apoio e prestabilidade quando precisei de ajuda, e também pelo companheirismo e constante boa disposição.

Quero deixar um especial agradecimento:

Ao João Couto e Joana Liberal por todas as dicas e conselhos uteis que me deram, e por andarem sempre a espalhar bom humor;

À Mónica Zuzarte e à Cátia Sousa tenho de agradecer por várias vezes me terem disponibilizado o material de que precisei, partilhado comigo os seus conhecimentos e pelo jeito autoritário, que no fundo não deixava de ser preocupação, com que se certificavam que eu executasse corretamente as minhas tarefas;

À Margarida Neves pela amizade, e por ter sido a minha companheira dos blots. Foste uma grande ajuda quando tudo era novo para mim, e sem dúvida que a aprendizagem não teria sido tão rápida sem ti.

À Isabel Ferreira tenho de agradecer pois foi ela quem me recebeu, introduziu, ensinou e acompanhou durante o meu percurso neste laboratório. Por me teres ajudado dar a volta quando os resultados pareciam maus quando na verdade era eu que precisava de ter uma perspetiva mais critica sobre eles, pelo tempo e paciência que tiveste comigo quando eu insistia na mesma tecla porque não conseguia entender, pela simpatia, disponibilidade e constante boa disposição.

Não posso deixar passar o meu profundo agradecimento ao Pedro Fernandes e à minha conterrânea Sónia Ferreira, meus colegas e amigos da UA, com quem tive a honra e enorme prazer de conjuntamente trabalhar, discutir ideias e resultados, explorar pontos de vista e fazer soar os alarmes do laboratório a altas horas da madrugada. Por me terem ajudado tão prontamente sempre que precisei de algum favor, pelos bons e divertidos momentos que partilhamos, pelo companheirismo, cumplicidade, apoio e amizade de curta data mas grande dimensão, um grande obrigado de coração.

Quero agradecer ainda aos meus colegas de mestrado Inês Barata, Luís Martinho, João Calmeiro, Jorge Silva, Helena Pereira, Vânia Vieira, Joana Martins e também, à nossa efémera colega por umas horas Patrícia Pereira. Todos nós entrámos no mesmo barco e remámos juntos a para alcançar um objetivo comum. Graças a vocês nunca me faltou motivação para que em todos os finais de 6ª-feira e sábados inteiros fosse até à faculdade de sorriso estampado. Pelas maratonas de estudo e trabalhos de grupo debaixo de pressão, mas também momentos de loucura e de diversão, pela cumplicidade, apoio e amizade, pelos sorrisos, chatices, alegrias, tristezas e todas as vivências partilhadas, um obrigado a todos os meus camaradas.

Quero ainda salientar a minha companheira Joana Martins com quem partilhei grande parte do tempo nesta reta final. A empatia contigo foi imediata desde início e apesar de umas vezes estar mais presente, outras vezes menos, contigo posso sempre contar. Pela tua prontidão, pelo teu sorriso rasgado, pela valiosa companhia nesta última maratona de trabalho por toda ajuda e favores que nunca recusaste, quero-te agradecer especialmente.

Devo também um profundo agradecimento aos meus amigos do ginásio Mauro Coelho, Priscila Santos, Ema Monteiro, Inês Lopes, Tatiana Pires, Fábio Teixeira, Rita Montez, Ana Nunes, Lucas Roos, Margarida Azenha, Sónia Russo e Thomas Martins, por fazerem desse espaço um excelente refúgio quando precisei de descomprimir. Por me terem promovido a

"quase staff", por todas atividades e vivências que partilhámos, pelo carinho, preocupação, interesse e apoio, no fundo por se terem tornado numa segunda família, um happy obrigado.

Não posso deixar de expressar o meu especial agradecimento ao Lucas Roos, à Margarida Azenha e à Sónia Russo, por terem sido companheiros constantes nesta Coimbra deserta de Agosto. Pelas bochechas e barriga doridas da compulsividade das nossas gargalhadas, pela simpatia, amizade, preocupação, motivação, prestabilidade e confiança, um sentido obrigado.

Ao meu grande amigo "Zé Master" Thomas Martins, por todas as conversas parvas e travessuras que engendrámos testando o limite da paciência das pessoas. Por seres das pessoas mais engraçadas, autênticas e bem-dispostas que conheço, por perguntares constantemente como corre o meu trabalho, por não me deixares desamparado e pela prontidão com que te dispões a ajudar, pela amizade, camaradagem e incentivo, por seres como um irmão, mes plus sincères remerciements.

Por último quero agradecer à minha família e em especial à minha mãe e irmã, pelo apoio, preocupação e carinho incondicionais. Pelo incentivo, coragem, paciência e acompanhamento nos momentos bons e menos bons. Por todo o esforço, empenho, dedicação e por sempre acreditarem em mim. Por serem os alicerces da pessoa que me tornei, a elas dedico todo este trabalho.

Apesar de ainda pequenininhas, quero expressar a minha gratidão para com as minhas sobrinhas pela pureza e genuinidade do amor com que me presenteiam. Um dia compreenderão esta mensagem.

A todos o meu muito obrigado!

LIST OF RELATED PUBLICATIONS

Papers in international scientific periodicals with referees

Catarino MD, Silva AM, Saraiva S, Sobral AJFN, Cardoso SM. Characterization of phenolic constituents and evaluation of antioxidant properties of leaves and stems of *Eriocephalus africanus*. *Arabian J Chem* DOI: 10.1016/j.arabjc.2015.04.018.

Papers in conference proceedings

Catarino MD, Silva J de A, Sobral, AJFN, Cardoso SM (2014). *Eriocephalus africanus*: derivados de ácido cafeico e suas potencialidades. Atas do XII Encontro de Química dos Alimentos (ISBN: 978-989-98541-6-1) p. 23-25.

Abstracts in international scientific periodicals with referees

Catarino MD, Silva JD, Sobral, AJFN, Cardoso SM (2014). Novel phenolics in *Eriocephalus* genus. *Planta Medica*, 80 (16), P1L130.

Santos SF, Catarino MD, Marcos M, Ferreira FM, Sobral AJFN, Cardoso SM (2013). Antioxidant properties of *Geranium robertianum* L. *Eur J Clin Invest* 43(Supl1), 62.

Saraiva SC, Catarino MD, Marcos M, Sobral AJFN, Cardoso SM (2013). *Eriocephalus africanus*: a potential source of antioxidant compounds. *Eur J Clin Invest* 43(Supl1), 62-63.

Resumo

Introdução: As plantas medicinais têm vindo a ser reconhecidas e utilizadas ao longo da história da humanidade pelos seus efeitos terapêuticos e curativos. Estas plantas conseguem sintetizar uma grande variedade de fitoquímicos biologicamente ativos, dos quais se podem destacar os compostos fenólicos. Visto que estes constituintes têm sido intimamente associados a propriedades benéficas para a saúde, um crescente interesse tem-lhes sido prestado por parte de vários investigadores. Dentro dos diferentes benefícios atribuídos aos compostos fenólicos, podem destacar-se as atividades antioxidantes e anti-inflamatórias. De acordo com o que tem vindo a ser descrito, estes compostos conseguem atenuar os efeitos negativos em condições de stress oxidativo através de mecanismos de sequestro de diferentes espécies reativas e atividade quelante de metais pesados, bem como intensificação da atividade de várias enzimas antioxidantes endógenas tais como a superóxido dismutase (SOD), glutationa peroxidase (GSH-px) e a catalase (CAT), ou ainda inibindo vias de sinalização pró-inflamatórias que incluem o óxido nítrico ($NO^{\bullet}$), oxido nítrico sintase induzível (iNOS), cicloxigenase-2 (COX-2), lipoxigenase (LOX) e fator nuclear-κB (NF-κB).

Eriocephalus africanus e *Geranium robertianum* são duas espécies de plantas que têm sido muito utilizadas na medicina tradicional devido às suas aclamadas propriedades benéficas. Como tal, neste trabalho pretendeu-se avaliar as suas capacidades antioxidantes e anti-inflamatórias, com o intuito de perceber se existe potencial para que estas plantas possam vir a ser exploradas com fins terapêuticos ou como formulações para nutracêuticos.

Métodos: As propriedades antioxidantes dos extratos hidroetanólicos das folhas e caules de *Eriocephalus africanus* (EAL e EAS, respetivamente) e *Geranium robertianum* (GRL e GRS, respetivamente) foram avaliadas pelos métodos $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $OH^{\bullet}$, $NO^{\bullet}$, TBARS, FRAP e ORAC. Uma vez que estes produtos podem vir a ser integrados na dieta humana, possíveis efeitos citotóxicos foram avaliados em linhas celulares de hepatócitos. Para a análise das propriedades anti-inflamatórias dos extratos, testaram-se as amostras investigando os seus efeitos inibidores da atividade da 5-LOX, através da medição espectrofotométrica da taxa de oxidação do linoleato, e produção de $NO^{\bullet}$ pelos macrófagos RAW 264.7 usando o método de *Griess*. A concentração de amostra que revelou uma melhor inibição dos níveis de nitritos foi também testada no que diz respeito aos seus efeitos sobre as enzimas ativadas durante a inflamação, nomeadamente iNOS e COX-2, através do método de Western blot recorrendo a anticorpos específicos. A identificação e quantificação dos compostos fenólicos de cada extrato foram conseguidas recorrendo ao método de Folin-Ciocalteu e através da análise por HPLC-DAD-ESI/MS^n, respetivamente.

<u>Resultados:</u> A atividade antioxidante mais promissora foi registada para o extrato GRL, tendo obtido os menores valores de IC_{50} para quase todos os ensaios (7.6±0.6, 3.9±0.6, 45.1±2.4, 20.0±0.9, 115.8±16.1 and 63.3±5.4 µg/mL para DPPH•, ABTS•+, OH•, NO•, TBARS e FRAP, respetivamente). Verificou-se uma exceção no método ORAC no qual o extrato EAS demonstrou possuir o dobro do poder antioxidante das restantes amostras (4.01±0.3 µM ET). Numa perspetiva geral, a segunda amostra mais promissora foi o extrato GRS seguido do extrato EAS. Globalmente, o extrato EAL foi o que obteve resultados menos promissores, e por isso foi excluído das análises posteriores. Nenhum dos extratos de *G. robertianum* e EAS revelou hepatotoxicidade sugerindo que as concentrações usadas (25 – 100 e 50 – 200 µg/mL, respetivamente) são inócuas. Na concentração de 75 µg/mL, ambos os extratos de *G. robertianum* inibiram aproximadamente 65% da atividade da 5-LOX enquanto o extrato EAS demonstrou uma inibição inferior (30%) para a mesma concentração. Mais ainda, este último extrato não revelou efeito na produção de nitritos em macrófagos estimulados com LPS para concentrações de 50 – 200 µg/mL. Por outro lado, ambos os extratos de *G. robertianum*, numa concentração de 100 µg/mL, demonstraram efeito inibidor na produção de NO• por parte dos macrófagos estimulados com LPS. No entanto, para esta mesma concentração verificou-se um decréscimo da viabilidade nas células tratadas com o extrato GRL, pelo que apenas o extrato GRS foi sujeito à análise por Western blot. No entanto, os dados recolhidos permitiram concluir que o extrato GRS (a 100 µg/mL) não causou inibição significativa nos níveis de iNOS ou COX-2.

Adicionalmente, a quantificação do teor total de compostos fenólicos de todos os quatro extratos revelou que a amostra com maior abundância destes compostos foi o extrato GRL (462 mg EAG/g de matéria seca), seguido pelos extratos EAS>GRS=EAL. Apesar das ligeiras variações nas intensidades dos picos, os perfis cromatográficos entre extratos da mesma planta revelaram-se semelhantes. Os extratos de *E. africanus* são particularmente ricos em ácidos clorogénicos e seus derivados, sendo que os dois maiores picos de ambos os extratos desta planta correspondem aos ácidos 3-cafeioilquínico e 3,5-dicaffeioilquínico. Um terceiro pico demonstrou-se relevante apenas no extrato EAL, tendo sido identificado como eriodictiol-hexuronídeo. Outros compostos minoritários foram também identificados nestes extratos, nomeadamente derivados de hesperetina, eriodictiol, ácidos cafeico, ferúlico e protocatecuico. Por outro lado, os extratos de *G. robertianum* revelaram-se mais enriquecidos em derivados de ácido elágico e gálico. Os três maiores picos destes extratos corresponderam a ácido carboxílico de brevifolina, ácido elágico e galoil-HHDP-hexosídeo. O ácido cafeioilquínico também foi detetado nos extratos de *G. robertianum*, bem como o ácido 3,4-dihidroxifenilacético, embora tenham sido considerados como compostos minoritários.

Conclusões: Globalmente, os extratos de ambas as plantas *E. africanus* and *G. robertianum* exibiram boas propriedades antioxidantes, sendo que o extrato de GRL demonstrou ser o mais promissor entre os quatro, provavelmente pelo seu teor mais elevado em compostos fenólicos. Além disso, apenas os extratos de *G. robertianum*, particularmente o extrato GRS, revelaram uma significativa capacidade inibidora da produção de nitritos pelos macrófagos RAW 264.7. Esta inibição não foi no entanto acompanhada pela diminuição nos níveis intracelulares de iNOS e/ou COX-2, presumindo-se assim que as propriedades anti-inflamatórias deste extrato são resultado apenas dos seus mecanismos de sequestro de radicais que são produzidos durante a inflamação.

Palavras chave: Plantas medicinais, nutracêutivos, *Eriocephalus africanus*, *Geranium robertianum*, stress oxidativo, inflamação, compostos fenólicos, ácido elágico, ácido gálico, ácido clorogénico

Abstract

Introduction: Medicinal plants have been identified and used throughout human history because of their therapeutic and healing effects. These plants can synthesize a wide variety of biological active phytochemicals from which phenolic compounds can be distinguished. Because these compounds have been closely associated with health promoting effects, they have drawn researchers increasing attention. Among the various health benefits attributed to the phenolic compounds, their antioxidant and anti-inflammatory properties have gathered particular interest. It has been described that they can effectively attenuate the oxidative stress imbalance through the scavenging of different reactive species and heavy metal chelating mechanisms, as well as enhancing the endogenous antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidade (GSH-px), and catalase (CAT), or inhibiting pro-inflammatory signaling pathways and mediators including nitric oxide ($NO^•$), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), lipoxygenase (LOX) and nuclear factor- κB (NF-κB).

Eriocephalus africanus and *Geranium robertianum* are two plant species that have been long used in traditional medicine due to their claimed beneficial properties. Therefore, this work intended to evaluate the safety profile of the hydroethanolic extracts obtained from *Eriocephalus africanus* and *Geranium robertianum* to human cells, their antioxidant and anti-inflammatory abilities, and to disclose the mechanism of action behind its bioactivity in order to understand if there is potential to exploit these plants for putative therapeutic uses or nutraceutical formulations.

Methods: The antioxidant properties of the hydroethanolic extracts from leaves and stems of *Eriocephalus africanus* (EAL and EAS, repectively) and *Geranium robertianum* (GRL and GRS, respectively) were evaluated through $DPPH^•$, $ABTS^{•+}$, $OH^•$, $NO^•$, TBARS, FRAP and ORAC assays. Since these products can be used in human diet, possible cytotoxic effects on hepatocytes were also evaluated. To analyze the anti-inflammatory potential of the extracts, samples were tested for their ability to inhibit 5-LOX activity, through the spectrophotometric measurement of the rate of linoleate oxidation, and the $NO^•$ release by macrophages, through the *Griess* method. The sample conditions that revealed inhibition of the $NO^•$ levels were also evaluated with regard to effects on the levels of intracellular enzymes triggered during inflammation, namely iNOS and COX-2, through Western blot using specific antibodies. Quantification and identification of the phenolic compounds of each extract were also achieved through Folin-Ciocalteu and HPLC-MS^n analysis, respectively.

<u>Results:</u> The most promising antioxidant activity was registered for the GRL extract, which had the lowest IC_{50} values for almost every tests (7.6±0.6, 3.9±0.6, 45.1±2.4, 20.0±0.9, 115.8±16.1 and 63.3±5.4 µg/mL for DPPH•, ABTS•+, OH•, NO•, TBARS and FRAP assays, respectively). The only exception was in ORAC assay where the EAS extract has shown twice the antioxidant power of the remaining samples (4.01±0.3 µM TE). From a general perspective, the second most promising sample was the GRS extract followed by the EAS extract. Overall, EAL extract was the less promising extract, and therefore it was excluded from the subsequent studies. No hepatotoxic effects were noted for both *G. robertianum* and EAS extracts, suggesting that they are safe for the concentrations used (25 – 100 and 50 – 200 µg/mL, respectively). Both *G. robertianum* extracts at 75 µg/mL inhibited approximately 65 % the 5-LOX activity, while a moderate inhibition (30%) was noted for the EAS extract at the same concentration. Moreover, no effects on the NO• release by LPS-activated macrophages were evidenced for the latter extract, up to the concentrations 50 – 200 µg/mL. On the other hand, both *G. robertianum* extracts inhibited NO• release evoked by LPS-stimulated macrophages for the highest concentrations tested (100 µg/mL). Despite this, in GRL-treated cells, this concentration was also responsible for the decrease of their viability and therefore only GRS extract was submitted to Western blot analysis. The gathered results allowed to observe however, that the treatment of LPS-stimulated RAW 264.7 with 100 µg/mL of GRS extract did not induce significant inhibition of iNOS or COX-2.

Additionally, the total phenolic quantification of the four extracts revealed that the most phenolic-enriched sample was the GRL extract (462 mg GAE/g of dry material), followed by EAS>GRS=EAL. Despite the slight variances in the peak intensities, the chromatographic profile between the extracts from the same plants were similar. *E. africanus* extracts were particularly rich in chlorogenic acid derivatives. The two main peaks identified in both samples correspond to 3-caffeoylquinic and 3,5-dicaffeoylquinic acids. A third relevant peak was also found in the EAL extract and was identified as eriodictyol-hexuronide. Other minor compounds namely hesperetin, eriodictyol caffeoyl, ferulic and protocatechuic derivatives have been identified as well. On the other hand *G. robertianum* extracts were more abundant in ellagic and gallic acid derivatives. The three main peaks disclosed correspond to brevifolin carboxilic acid, ellagic acid and galloyl-HHDP-hexoside. Caffeoylquinic acid was also present in these samples alongside with 3,4-dihydroxyphenylacetic acid, which have only been detected as minor compounds.

<u>Conclusion:</u> Overall, both *E. africanus* and *G. robertianum* exhibited good antioxidant properties, but GRL extract was shown to be more effective than the others probably due to its higher content in phenolics. Moreover, only *G. robertianum* extracts, in particularly GRS

have exerted significant inhibition of NO• production, but since no ingibitory effects have been observed in the levels of intracellular iNOS and COX-2, it is suggested that this extract may exert its anti-inflammatory abilities through the scavenging of the reactive species generated during inflammation.

Keywords: Medicinal plants, nutraceuticals, *Eriocephalus africanus*, *Geranium robertianum*, oxidative stress, inflammation, phenolic compounds, ellagic acid, gallic acid, chlorogenic acid

Table of Contents

Resumo I

Abstract IV

Table of Contents IX

List of Figures XIII

List of Tables XVII

List of Abbreviations XVIII

1 INTRODUCTION 1

1.1 Medicinal plants 3

1.2 Phenolic compounds 3

1.2.1 Polyphenols chemistry 6

1.2.1.1 Flavonoids 6

1.2.1.2 Isoflavonoids, neoflavonoids, chalconoids and auronoids 9

1.2.1.3 Non-flavonoids 9

1.2.1.4 Stilbenes and Lignans 10

1.2.2 Bioavailability of phenolic compounds 10

1.2.3 Oxidative stress and inflammatory conditions 12

1.2.3.1 Involvement of polyphenols in oxidative stress and inflammation 14

1.3 *Eriocephalus africanus* L. and *Geranium robertianum* L. 16

2 OBJECTIVES 21

3 EXPERIMENTAL PROCEDURE 25

3.1 Chemicals 27

3.2 Plant material 27

3.3 Extraction of phenolic compounds 27

3.4 Antioxidant properties 28

3.4.1 $DPPH^{\bullet}$ scavenging assay 28

3.4.2 $ABTS^{\bullet+}$ decolorization assay 28

3.4.3 $OH^{\bullet}$ scavenging assay 29

3.4.4 Lipid peroxidation inhibitory capacity in the presence of thiobarbituric acid reactive substances (TBARS) assay 30

3.4.5 Oxygen radical absorbance capacity (ORAC) assay 31
3.4.6 Chemical NO• scavenging assay 32
3.5 Evaluation of the hepatocytes viability 33
3.5.1 Cell culture 33
3.5.2 Hepatocytes viability by the MTT assay 33
3.6 Anti-inflammatory properties 33
3.6.1 Cell culture 33
3.6.2 Cell viability by MTT assay 34
3.6.3 Inhibition of cellular NO• production 34
3.6.4 Soybean 5-Lipoxygenase (5-LOX) assay 35
3.6.5 Cell lysates and Western blot analysis for measurement of iNOS and COX-2 levels 35
3.7 Quantification and identification of phenolic compounds through HPLC–DAD–ESI/MS^n 36
3.8 Statistical analysis 38
4 RESULTS 39
4.1 Determination of the antioxidant activities 41
4.1.1 DPPH•, ABTS•+ and HO• scavenging activities 41
4.1.2 Ferric reducing antioxidant power and lipid peroxidation inhibitory capacities 43
4.1.3 Oxygen radical absorbance capacity 44
4.1.4 NO• scavenging activity in chemical model 45
4.2 Evaluation of hepatocytes viability 46
4.3 Determination of anti-inflammatory activities 47
4.3.1 Effects on soybean 5-Lipoxygenase 47
4.3.2 Effects on NO• production and cell viability on macrophages RAW 264.7 48
4.3.3 Effects on expression of iNOS and COX-2 enzymes 50
4.4 Identification and quantification of phenolic constituents from E. africanus and G. robertianum 51
4.4.1 Phenolic constituents in *E. africanus* extracts 52
4.4.2 Phenolic constituents of *G. robertianum* extracts 55
5 DISCUSSION 59

6 CONCLUSIONS AND FUCTURE PERSPECTIVES 67

7 REFERENCE LIST 751

List of Figures

FIGURE 1 – Number patents registered in the 2005 - 2015 period by simple search in WIPO using the keywords "Polyphenols" and “Food Supplement” 6

FIGURE 2 – Schematic structure of the flavan nucleus, the basic flavonoid skeleton............... 6

FIGURE 3 – Representation of the major classes of flavonoids 7

FIGURE 4 – Representation of the six different sub-classes of flavonoids 7

FIGURE 5 – Representation of the backbone structure of the three main non-flavonoid classes 10

FIGURE 6 – Representation of the trajectory of polyphenols and metabolites in human’s organism 11

FIGURE 7 – Overview of the signaling cascades that mediate the oxidative stress and inflammatory conditions.. 13

FIGURE 8 –*Eriocephalus africanus* L. (A) and *Gernaium robertianum* L. (B).............................. 16

FIGURE 9 – Approach to the geographical distribution of E. africanus (A) and G. robertianum (B) around the world. This maps are based on occurrence records available through the GBIF Backbone Taxonomy, 2013-07-01 and may not represent the entire distribution. Accessed via http://www.gbif.org/species/2890668 on 2015-07-16 17

FIGURE 10 – Number of publications in the 1995 - 2014 period by simple search in ISI Web of Science® using the keywords "*Eriocephalus africanus*" (A) and “*Geranium robertianum*” (B)............. 18

FIGURE 11 – Plots of Trolox Kinetic Curves. Representative curves from ORAC assay of varying concentrations of Trolox antioxidant standards ranging from 0 to 25 µM. 31

FIGURE 12– Dose-dependent antioxidant activity curves obtained for the leaves and stems extract of *E. africanus* (EAL and EAS, respectively) and *G. robertianum* (GRL and GRS, respectively) estimated by the $DPPH^{\bullet}$ (A), $ABTS^{\bullet+}$ (B) and $OH^{\bullet}$ (C) scavenging assays. The results shown were obtained from the mean of at least three replicates. 41

FIGURE 13 – Representation of the IC_{50} values determined for the various samples and standard compounds tested in the $DPPH^{\bullet}$ (A), $ABTS^{\bullet+}$ (B) and $OH^{\bullet}$ (C) scavenging methods. Ascorbic acid was the standard compound (STD) used in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, while mannitol was the standard compound used in $OH^{\bullet}$ scavenging. Statistical analysis was performed by one-way ANOVA, followed by Tukey’s post-hoc test. ***P<0.001; **P<0.01, compared to the STD. Data represent mean ± SEM of 3 independent assays 42

FIGURE 14 – Dose-dependent antioxidant activity curves obtained for leaves and stems extract of *E. africanus* (EAL and EAS, respectively) and *G. robertianum* (GRL and GRS, respectively), estimated by the FRAP (A) and TBARS (B) assays. The results shown were obtained from the mean of at least three replicates.. 43

FIGURE 15 – Representation of the IC_{50} values determined for the various samples and standard compounds tested in the FRAP (A) and TBARS (B) assays. BHT was the standard compound (STD) used in FRAP, while trolox was the standard compound used in TBARS. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001, compared to the STD. Data represent mean ± SEM of 3 independent assays 44

FIGURE 16 – Dose-dependent antioxidant curves obtained for the leaves and stems extracts of *E. africanus* and *G. robertianum* estimated through the ORAC assay. The results shown were obtained from the mean of at least three replicates. 45

FIGURE 17 – Dose-dependent antioxidant response curves obtained for *E. africanus* extracts (EAL and EAS, respectively) (A) and *G. robertianum* extracts (GRL and GRS, respectively) (B) estimated through the $NO^{\bullet}$ scavenging assay. The corresponding IC_{50} values are displayed in (C). The standard compound (STD) used was ascorbic acid. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001, compared to the STD. Data represent mean ± SEM of 3 independent assays 46

FIGURE 18 – Cell viability of HepG-2 cells treated with GRS (A), GRL (B) (25, 50, 75, and 100 µg/mL) and EAS extracts (C) (50, 75, 100 and µg/mL). Cell viability was assessed using the MTT assay and the results were expressed as percentage of control cells (Ctrl). Each value represents the mean ± SEM of at least 3 independent experiments. 47

FIGURE 19 – Kinetic curves of the 5-LOX activity in absence or presence of 75 µg/mL of the samples studied (A) and their correspondent percentages of inhibition (B). The standard compound (STD) used was ascorbic acid. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001, compared to the control (Ctrl). Data represent mean ± SEM of 3 independent assays. 48

FIGURE 20 – Effects of the pre-treatment with EAS (50, 75, 100 and 200 µg/mL), GRS and GRL (25, 50, 75 and 100 µg/mL) on the cell viability (% of the control) (A, B and C, respectively) and $NO^{\bullet}$ levels (µM) (D, E and F, respectively) on RAW 264.7 cells after 24h of incubation with (■) or without (□) LPS. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. **P<0.01; ***P<0.001, compared to the control without LPS; #P<0.05; ##P<0.01; ###P<0.001, compared to the control with LPS. Data represent mean ± SEM of 3 independent assays. 49

FIGURE 21 – Effect of the GRS extract (100µg/L) pre-treatment in the expression of iNOS and COX-2 enzymes (% of the LPS) in RAW 264.7 macrophages after 24h of incubation with (■) or without (□) LPS. An anti-β-Tubulin antibody was used to confirm equal protein loading and normalize the data. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001, compared to the control without LPS; #P<0.05; ###P<0.001, compared to the control with LPS. The blot is representative of 3 similar blots. iNOS=135 kDa and COX-2=69 kDa 50

FIGURE 22 – Quantification of the total phenolic content on *E. africanus* leaves and stems (EAL and EAS, respectively) and *G. robertianum* leaves and stems extracts (GRL and GRS, respectively).

Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001; **P<0.01, compared to GRL. Data represent mean ± SEM of 3 independent assays. 51

FIGURE 23 – Chromatographic profile of *E. africanus* hydroethanolic extracts at 280 nm. Chromatogram corresponding to stems extract is represented in bold lines, while the thin lines represent the chromatogram corresponding to the leaves extract. QA – quinic acid; PCA-hex – protocatechuic acid-hexoside; 1-CQA – 1-caffeoylquinic acid; FA-der – ferulic acid derivative; ERD-hexa – eriodictyol-hexuronide; CA-der – caffeic acid derivative; 3,4-diCQA – 3,4-dicaffeoylquinic acid, 3,5-diCQA – 3,5-dicaffeoylquinic acid; 4,5-diCQA – 4,5-dicaffeoylquinic acid; ERD – eriodictyol. 52

FIGURE 24 – Chromatographic profile of *G. robertianum* hydroethanolic extracts at 280 nm. Chromatogram corresponding to stems extract is represented in bold lines, while the thin lines represent the chromatogram corresponding to the leaves extract. DOPAC – 3,4-dihydroxyphenylacetic acid; GA – gallic acid; CQA – caffeoylquinic acid; ET-der – ellagitannin derivative; G-HHDP-hex – Galloyl-HHDP- hexoside; BCA – brevifolin carboxylic acid; EA – ellagic acid. 55

FIGURE 25 – Proposal of the anti-inflammatory mechanism of the GRS extract in the pathophysiology of inflammation. The oxidative stress stimulus trigger the activation of nuclear factor κB (NF-κB) that translocates to the nucleus where it enhances the transcription of various pro-oxidant and pro-inflammatory mediators genes including those of the enzymes nicotinamide adenine dinucleotide phosphate oxidase (NOX), xanthine oxidase (XOX), cyclooxygenase-2 (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS), as well as several cytokines and chemokines. The pro-oxidant enzymes together with mitochondrial activity will generate more reactive species that will contribute for the increase of the oxidative stress. In the presence of the GRS extract the reactive species are neutralized attenuating the oxidative stress condition. In turn, the three pro-inflammatory enzymes produce prostaglandins (PGE_2) leukotrientes (LT_4) and nitric oxide that are released in the extracellular space together with various cytokines and chemokines propagating the inflammation by transducing the signals to other cells. In presence of the GRS extract, the levels of nitric oxide produced by iNOS are reduced due to its ability to scavenge this radical. Likewise, the GRS extract interferes with the LOX activity reducing the release of leukotrienes to the extracellular space, contributing for the attenuation of the inflammatory event. 70

List of Tables

TABLE 1 – Polyphenols content in some common foods and their estimated daily intake*..... 5

TABLE 2 – Linearity, LOD and LOQ of four standard compounds used as reference 38

TABLE 3 – ORAC values obtained for the four samples tested 45

TABLE 4 – Identification of LC-DAD-ESI/MS^n data, and quantification of the most relevant fractions from the extracts of *E. africanus*. 54

TABLE 5 – Identification of LC-DAD-ESI/MS^n data of the most relevant fractions from the extracts of *G. robertianum*. 56

List of Abbreviations

5- LOX
  5-Lipoxygenase
AAPH
  2,2'-azobis(2-amidino-propane) dihydrochloride,
$ABTS^{\bullet+}$
  2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid),
$ABTS\text{-}NH_4$
  2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt,
AUC
  Area under the curve,
BHT
  Butylated hydroxytoluene,
CAT
  Catalase,
CID
  Collision-induced dissociation,
CO_2
  Carbon dioxide,
COX-2
  Cyclooxygenase-2,
DAD
  Diode array detector,
DMEM
  Dulbecco's modified eagle medium,
DNA
  Deoxyribonucleic acid,
$DPPH^{\bullet}$
  2,2-diphenyl-1-picrylhydrazyl radical,
DTT
  Dithiothreitol,
EAL
  E. africanus leaves,
EAS
  E. africanus stems,
ECF
  Enhanced chemifluorescence,
EDTA
  Ethylenediamine tetraacetic acid,
ESI
  Electrospray ionization,
GAE
  Gallic acid equivalents,
FBS
  Fetal bovine serum,
Fe^{2+}
  Ferrous cation,
Fe^{3+}
  Ferric cation,
$FeCl_3$
  Iron chloride,
FRAP
  Ferric reducing power assay,

GRL
  G. robertianum leaves,
GRS
  G. robertianum stems,
GSH-px
  Glutathione peroxidase,
H_2O_2
  Hydrogen peroxide,
HAT
  Hydrogen electron transfer,
HCl
  Hydrochloric acid,
HepG-2
  Human hepatic carcinoma cell line,
HOCl
  Hypochlorous acid,
HPLC
  High performance liquid chromatography,
IκB
  Inhibitor of κB,
iNOS
  Inducible nitric oxide synthase,
LPS
  Lipopolysaccharide,
MDA
  Malondialdehyde,
MS
  Mass spectrometer,
MTT
  3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide,
NaCl
  Sodium chloride,
NaOH
  Sodium hydroxide,
NF-κB
  Nuclear factor-κB,
NO
  Nitric oxide,
$NO^{\bullet}$
  Nitric oxide radical,
NO_2^-
  Nitrite ion,
NOX
  Nicotinamide adenine dinucleotide phosphate oxidase,
$O_2^{\bullet-}$
  Superoxide anion,
$OH^{\bullet}$
  Hydroxyl radical,
ORAC
  3.5.6. Oxygen radical absorbance capacity,
PBS
  Phosphate Saline Buffer,
PVDF
  Polyvinylidene difluoride,
RAW 264.7

Mouse leukaemic monocyte macrophage cell line,
RIPA
Radio-Immunoprecipitation Assay,
RNS
Reactive nitrogen species,
SET
Single electron transfer,
ROS
Reactive oxigen species,
SOD
Superoxide dismutase,
TBA
Thiobarbituric acid,
TBARS
Thiobarbituric acid reactive substances,
TBS-T
Tris – buffered saline containing 0.1% (v/v) Tween®
TCA
Trichloroacetic acid,
TE
Trolox equivalents,
UV/vis
Ultraviolet/visible,
wt
Weight,
XOX
Xanthine oxidase,

INTRODUCTION

1 INTRODUCTION

1.1 Medicinal plants

For a long time, medicinal plants have been used in traditional medicine with therapeutic purposes being recognized since ancient times for their healing effects. Indeed, natural products form a library of bioactive compounds that humankind has been using for the treatment of several pathologies. Approximately 66% of the new small-molecules and chemical entities introduced as drugs worldwide from 1981 to 2010 were derived from natural products. More than twenty three new drugs derived from natural sources have been launched on the market after 2000 and were approved for the treatment of cancer, neurological, infectious, cardiovascular, metabolic, immunological and inflammatory diseases (Cragg & Newman, 2013).

Towards the end of 20th century, epidemiological studies and associated meta-analyses strongly suggested that long term consumption of plants offered some health benefits including anti-allergic, anti-hypertensive, antimicrobial, antioxidant, anti-inflammatory, anti-cancer, anti-diabetes, anti-aging, cardio-protective and neuro-protective effects, thus suggesting that they may play an important role in the maintenance of the human health (Pandey & Rizvi, 2009). Since then, plant phytochemicals have increasingly attracted much scientific interest, and many *in vitro* and *in vivo* studies have described their extensive biological properties, highlighting their potential to be used with therapeutic purposes (Pereira et al., 2009; Puupponen-Pimiä et al., 2001).

1.2 Phenolic compounds

Among the extensive number of the existent phytochemicals, phenolic compounds (also known as phenolics or polyphenols) are the ones most ubiquitously distributed throughout the plant kingdom. In fact, this is the biggest group of phytochemicals, with more than 8,000 identified compounds (Seeram, 2010).

All polyphenols arise from phenylalanine through the phenylpropanoid pathway or from a close precursor, shikimic acid, constituting an important group of secondary metabolites which can be found in the vacuoles of flowers, leaves, stems and roots of both edible and non-edible plants (Ferreyra, Rius & Casati, 2012; Pandey & Rizvi, 2009). They serve vital roles in plants physiology, which include the response to abiotic stress conditions such as rainfall and ultraviolet light radiation, regulation of cell functions, reproduction and survival. (Daglia, 2012). Moreover, they can act either as attractants or repellants and/or cytotoxic agents, drawing

pollinators and symbionts, while deterring herbivores and pathogenic microorganisms (Dixon & Pasinetti, 2010).

Inevitably, since polyphenols are abundant in vegetables, fruits, nuts, seeds and consequent plant origin foods and beverages (e.g. tea, wine, olive oil and many others), they are deeply integrated in human daily diet (Catarino *et al.*, 2014). In fact, they are very important elements that contribute for the food quality, organoleptic and nutritional properties (Gharras, 2009). It is estimated that the average intake of total polyphenols is around 1 g/day, with flavonoids accounting for two thirds of this value and phenolic acids contributing for the remaining one third (Scalbert & Williamson, 2000). However, these values were suggested to be slightly underestimated due to insufficient data on the polyphenol content of foods (proanthocyanidins and thearubigins) (Lamport *et al.*, 2012). Table **1** describes the polyphenol content in some selected foods present in human daily diet.

When compared to pharmaceutical drugs, these metabolites have low potency as bioactive compounds, but since they are ingested regularly and in significant amounts as part of our diet, they may have a noticeable long-term physiological effect (Cilla *et al.*, 2013).

Because of that, in the past few years, new non-food products containing food extracts or phytochemical-enriched extracts to which a beneficial physiological function has been directly attributed have been created and commercialized in the form of pharmaceutical products, i.e., pills, capsules, solutions, gels, liquors, powders, granulates, etc. However, since these products cannot be truly classified as "food" and they are not pharmaceutical drugs either, a new hybrid term between nutrients and pharmaceuticals have emerged: "nutraceuticals". Despite the term still does not possess a clear definition, it is generally accepted as "diet supplements that deliver a concentrated form of a presumed bioactive agent from a food, presented in a non-food matrix, and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods" (Ansorena *et al.*, 2013). Ginkgo biloba, ginseng, ginger, milk thistle, soya, St. John's wort and valerian are well known examples of plants that are frequently used in different food supplements to enhance immunity, act as anti-inflammatory, anticancer, antidepressant and for the treatment of many disorders.

TABLE I – Polyphenols content in some common foods and their estimated daily intake*

	Food source	Polyphenol content (mg/kg fresh wt or mg/L)	Estimated daily intake (mg/day)
Flavonols:	Yellow onion	350-1200	
Quercetin	Curly kale	300-600	
Kaempferol	Leek	30-225	
Myricetin	Cherry tomato	15-200	
	Broccoli	40-100	
	Blueberry	30-160	13
	Black currant	30-70	
	Apricot	25-50	
	Apple	20-40	
	Beans, green or white	10-50	
	Black grape	15-40	
Flavones:	Parsley	240-1850	
Apigenin	Celery	20-140	1.6
Luteolin	Capsicum pepper	5-10	
Flavanones:	Orange juice	215-685	
Hesperetin	Grape fruit juice	100-650	14.4
Naringenin	Lemon juice	50-300	
Eriodictyol			
Isoflavones	Soy flour	800-1800	
Daidzein	Soybeans, boiled	200-900	
Genistein	Miso	250-900	USA/Netherlands: 1.2
Glycitein	Tofu	80-700	Asia: 25-50
	Tempeh	430-530	
	Soy milk	30-175	
Monomeric flavonols:	Chocolate	460-610	
Catechin	Green tea	100-800	
Epicatechin	Beans	350-530	
	Apricot	100-250	
	Cherry	50-220	
	Grape	30-175	156
	Peach	50-140	
	Blackberry	130	
	Apple	20-120	
	Black tea	60-500	
	Red wine	80-300	
Anthocyanins:	Aubergine	7500	
Cyanidin	Blackberry	1000-4000	
Pelargonidin	Black currant	1300-4000	
Peonidin	Blueberry	250-5000	
Delphinidin	Black grape	300-7500	
Malvidin	Cherry	350-4500	3.1
	Rhubarb	2000	
	Strawberry	150-750	
	Red wine	200-350	
	Plum	20-250	
	Red cabbage	250	
Phenolic acids:	Blackberry	80-270	
Protocatechuic acid	Raspberry	60-100	
Gallic acid	Black currant	40-130	
p-hydroxybenzoic acid	Strawberry	20-90	
Hydroxycinnamic acids:	Blueberry	2000-2200	Non coffee drinkers:
Caffeic acid	Kiwi	600-1000	< 25
Chlorogenic acid	Cherry	180-1150	Coffee drinkers:
Coumaric acid	Plum	140-1150	500 – 800
Ferulic acid	Aubergine	600-660	
Sinapic acid	Apple	50-600	
	Pear	15-600	
	Chicory	200-500	
	Artichoke	450	
	Potato	100-190	
	Corn flour	310	
	Flour: wheat, rice, oat	70-90	
	Cider	10-500	
	Coffee	350-1750	

*(adapted from Made, van der & Mensink, 2015 and Manach *et al.*, 2004)

Notably, polyphenols are frequently present as main ingredients in these supplements and some of the most commonly found in the nutraceutical market are anthocyanins, proanthocyanidins, flavonols, stilbenes, hydroxycinnamates, coumarins, ellagic acid and ellagitannins, isoflavones, lignans, etc. (Cilla *et al.*, 2013). As observable in the figure **1**, during the last decade, an average of 207 of new patents related to nutraceuticals containing polyphenols have been registered per year.

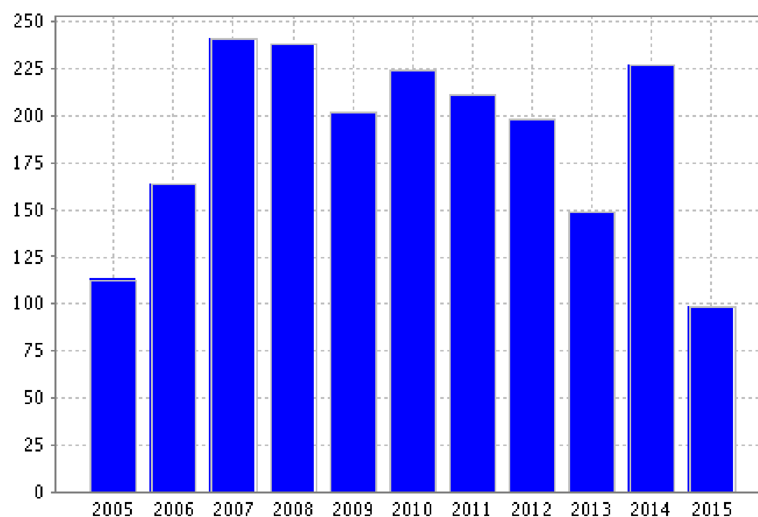


FIGURE 1 – Number patents registered in the 2005 - 2015 period by simple search in WIPO using the keywords "Polyphenols" and "Food Supplement"

1.2.1 Polyphenols chemistry

Two distinct groups of polyphenols can be denoted, i.e., flavonoids and non-flavonoids. In turn, these are divided in many classes and sub-classes which will be further discussed in below.

1.2.1.1 Flavonoids

Chemically, these compounds are low molecular weight polyphenolic substances based on a 15-carbon skeleton, consisting of two aryl rings (A- and B-rings) connected by an heterocyclic pyran ring (C-ring) and forming the C_6-C_3-C_6 flavan nucleus depicted in Fig. **2** (Petrussa *et al.*, 2013; Sandhar *et al.*, 2011).

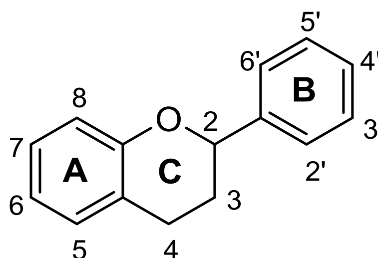


FIGURE 2 – Schematic structure of the flavan nucleus, the basic flavonoid skeleton

The position of the aryl substituent divides the flavonoid group into different classes, namely flavonoids (2-arylflavan), isoflavonoids (3-arylflavan) and neoflavonoids (4-arylflavan) (Fig. **3**). In addition, flavonoids may occur as flavan-opened chain compounds to give chalconoids, or as the 5-membered C-ring derivatives auronoids (Fig. **3**) (Iwashina, 2000).

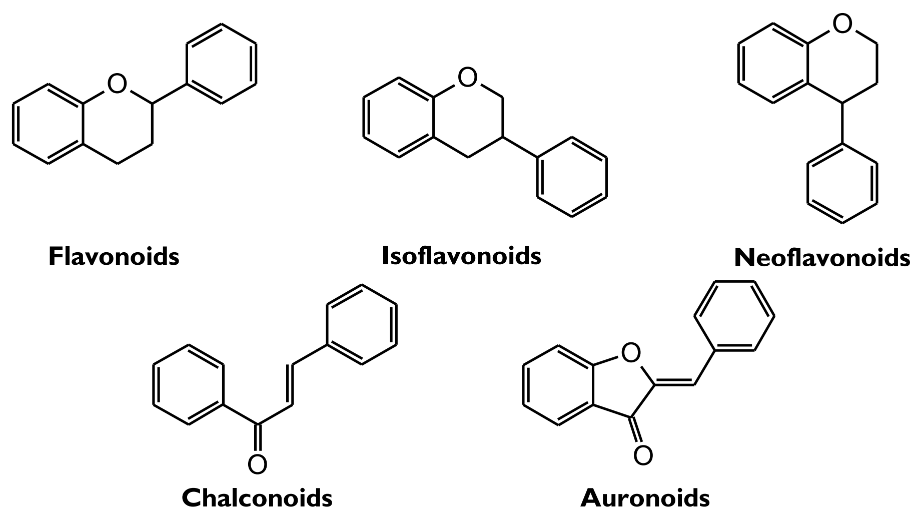


FIGURE 3 – Representation of the major classes of flavonoids

According to the degree of oxidation and substitution pattern of the heterocycle, i.e., the presence or absence of the ketone group and/or the 3-hydroxyl group in the C-ring, six different sub-classes of flavonoids can be distinguished: flavones, flavon-3-ols, flavan-3-ols (or chatechines), flavanones, dihydroflavon-3-ols (or flavanon-3-ols) and anthocyanins (Fig. **4**) (Marais *et al.*, 2006).

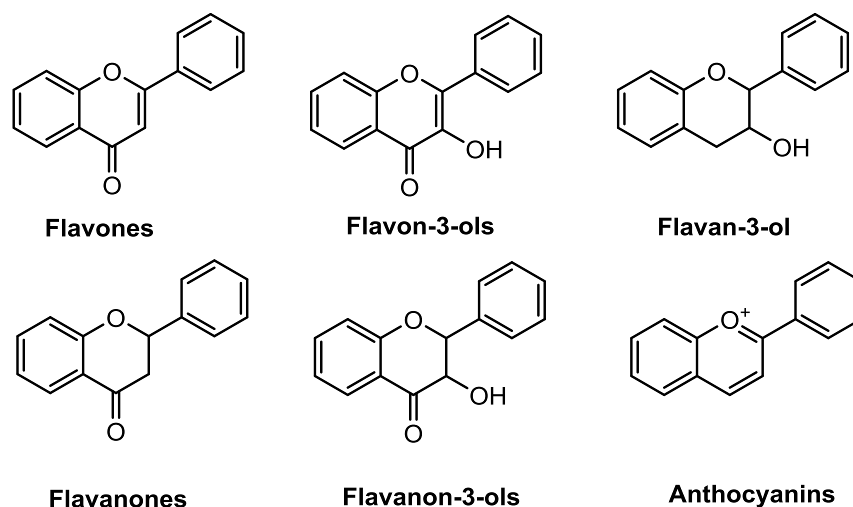


FIGURE 4 – Representation of the six different sub-classes of flavonoids

Individual compounds within each sub-class are resultant from further substitutions and arrangement of hydroxyl and/or methoxyl groups on the A- and B-rings (Kumar & Pandey, 2013). Naturally, flavonoids may occur individually as free aglycones (basic flavonoid structure), or modified by glycosylation or alkylation (Tanwar & Modgil, 2012). Most common *C*- and/or

O-glycosylation sites occur at C3, C5, C7, C3' and C4', and usually glucose, rhamnose, glucorhamnose, galactose and arabinose are the most frequently encountered sugar residues (Kumar & Pandey, 2013; Xiao, Muzashvili & Georgiev, 2014).

Flavones and flavon-3-ols, which differ from each other for the absence/presence of a 3-hydroxyl group in the pyrone ring, are the most abundant categories of flavonoids in nature (Comalada, Xaus & Gálvez, 2013). The most commonly found flavonol is quercetin, while luteolin and apigenin are the most widely distributed flavones, mainly in their glycoside forms (Gharras, 2009; Lamport *et al.*, 2012).

Flavanones and dihydroflavon-3-ols are the respective equivalents from the latter compounds with the particularity of the saturation of C_2–C_3 bond, i.e., they lack of the double bond between these two carbons on the pyrone ring. Hesperetin, naringenin and eriodictyol, are the three main flavanones in nature, and they can be chiefly found in citrus fruits (Tomás-Barberán & Clifford, 2000). On the other hand, the most well-known dihydroflavonol is taxifolin, though its appearance in nature is rather uncommon (Slimestad, Fossen & Vågen, 2007).

Flavan-3-ols have a skeleton similar to that of flavanon-3-ols, except for the absence of the 4-keto group (Comalada, Xaus & Gálvez, 2013). These compounds may exist in both the monomer (catechins) and polymer form (proanthocyanidins) and are among the most frequent flavonoids in our diet. Catechins may be found in many fruits, but it is undoubtedly in green tea where they are predominant (see table 1). As a result of the fermentative process applied for the production of black tea, the high levels of (gallo)catechins common in green tea decay to give way to the oxidized derivatives: theaflavins (dimers) and thearubigins (polymers) (Knaze *et al.*, 2012). On the other hand, proanthocyanidins, also known as condensed tannins, are complex catechins bound together by links between C4 and C8 (or C6), forming dimers, oligomers, and polymers which are responsible for the astringency of fruits and beverages (including grapes, persimmon, berries, wine, tea, etc) (Gu *et al.*, 2004; Hooper *et al.*, 2008). Procyanidins-rich products are one of the most common nutraceuticals in the market. The most popular are those based on grape seed extracts which are usually sold as '95% procyanidins standardized extracts' pills or capsules. The main activity attributed to theses nutraceuticals is their antioxidant activity (Espín, García-Conesa & Tomás-Barberán, 2007).

The remaining flavonoid sub-class are the anthocyanins which can be distinguished from the others due to their flavylium (2-arylchromenylium) ion skeleton that causes them to be highly colored (Jurd, 2013). Therefore, these compounds are pigments responsible for the red, blue or purple colors in fruit and flower tissues. Because their free aglycone forms are highly unstable, they always appear in their glycosylated form (Veberic *et al.*, 2015).

Together with the procyanidins, anthocyanins have occupied a prominent place in the market of products such as nutraceuticals and dietary supplements. The main dietary origin of these products is either single berry or natural combinations of various berries (blend of blueberry, strawberry, cranberry, wild bilberry, elderberry, and raspberry extracts). Often, they are also combined with other food components and are commercialized as powders, capsules, or tablets mainly claiming their high level of antioxidant capacity (Espín, García-Conesa & Tomás-Barberán, 2007).

1.2.1.2 Isoflavonoids, neoflavonoids, chalconoids and auronoids

Isoflavonoids, neoflavonoids, chalconoids and auronoids are much less common flavonoids compared to those previously mentioned. Isoflavonoids have gained increasing interest since they are important phytoestrogens which are believed to have several biological effects in humans via estrogen receptors. Because of that, this kind of polyphenols are frequently found in dietary supplements mainly related to menopausal women (North American Menopause Society, 2011).

Neoflavonoids, chalconoids and auronoids are classes of compounds that do not occur frequently in nature, although they have already been documented for a broad spectrum of health benefits (Garazd, Garazd & Khilya, 2003).

1.2.1.3 Non-flavonoids

Phenolic acids are non-flavonoid polyphenos which can be further divided into phenolic and hydroxycinnamic acid derivatives based on C_6–C_1 and C_6–C_3 backbones, respectively (see Fig. **5**). While fruits and vegetables contain many free non-flavonoids, in grains and seeds, these are often found in the bound form (Adom & Iu, 2002; Chandrasekara & Shahidi, 2010; Kim *et al.*, 2006). The phenolic acid content of edible plants is generally low. Besides, they are commonly present in the bound form and are typically components of complex structures like lignins and hydrolyzable tannins. They can also be found in the form of sugar derivatives and organic acids in plant foods (D'Archivio *et al.*, 2007; Liu, 2004).

Hydoxycinnamic acids are mainly found in nature in glycosylated derivatives or esters of quinic, shikimic or tartaric acid. These compounds have received increasing interest for their ability to inhibit the low-density lipoprotein (LDL) oxidation, among other biological properties (McCune *et al.*, 2011). Interestingly, the consumption habits of coffee has a serious impact in people's daily intake of hydroxycinnamates. A person who drinks three cups/day may ingest as much as 800 mg of hydroxycinnamic acid, whereas subjects who do not drink coffee and eat little amounts of fruits barely reach the 25 mg/day (Freitas, de, 2012).

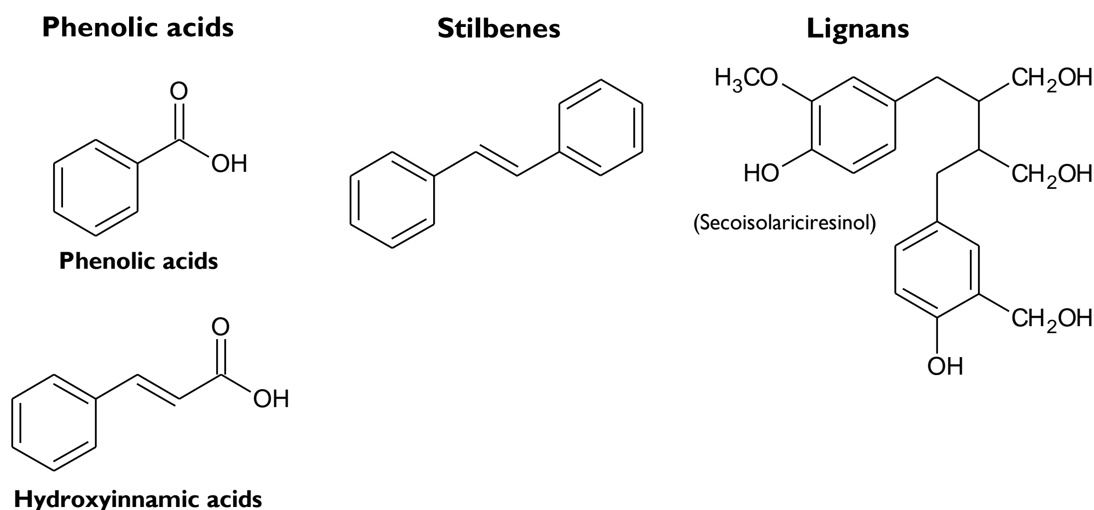


FIGURE 5 – Representation of the backbone structure of the three main non-flavonoid classes

1.2.1.4 Stilbenes and Lignans

Stilbenes are another class of phenolic compounds that is little widespread in plants. Nevertheless, extensive studies have been performed with resveratrol as it was found that it possesses potent anti-oxidant, anti-inflammatory, anti-obesity, anti-carcinogenic properties, and more recently it has shown the ability to activate sirtuins which have been suggested to mediate the lifespan extending effect of a low calorie diet, thus justifying its common use in several nutraceuticals (Gertz *et al.*, 2012; Silk & Smoliga, 2014).

Lignans are compounds highly present in flaxseed (mainly as secoisolariciresinol, Fig. **5**) that are metabolized by the intestinal microflora to produce "mammalian lignans" which are recognized as estrogen-like molecules and related to lower breast cancer risk (Carreau *et al.*, 2008).

1.2.2 Bioavailability of phenolic compounds

Polyphenols bioavailability and accumulation in body tissues is a very complex topic that has raised much debate within the scientific community and it is still not fully understood. The truth is that bioavailability differs greatly from one polyphenol to another, and the most abundant polyphenols in our diet are not necessarily those leading to the highest concentrations of active metabolites in target tissues (Manach *et al.*, 2005).

The absorption of food phenolics is primarily dictated by their chemical structure, as globally determined by several features (e.g. the degree of glycosylation, acylation, conjugation with other phenolics, molecular size, degree of polymerization and hydrophobicity) (Landete, 2012). Besides, the site of absorption also determines where some polyphenols may be better absorbed than others. Generally, flavonoid aglycones and specially hydroxycinnamic acids in

their free forms can be considerably absorbed from the small intestine (D'Archivio *et al.*, 2010). The exception are the anthocyanin glycosides which have been demonstrated to be quickly and efficiently absorbed in the stomach (Fernandes *et al.*, 2014). Other phenolics may be better absorbed in the large intestine or in the upper gastrointestinal tract after enzymatic hydrolysis or microbiota modification (D'Archivio *et al.*, 2010).

During the course of the absorption, polyphenols undergo extensive modification by methylation, sulfation and/or glucuronidation in the intestinal cells and later in the liver, representing a metabolic detoxification process, that facilitates their biliary and urinary elimination through increment of their hydrophilicity (Fig. **6**) (Crozier, Rio, Del & Clifford, 2010). As a consequence, the forms reaching the blood and tissues are different from those present in food, making it very difficult to identify all the metabolites and to evaluate their biological activity.

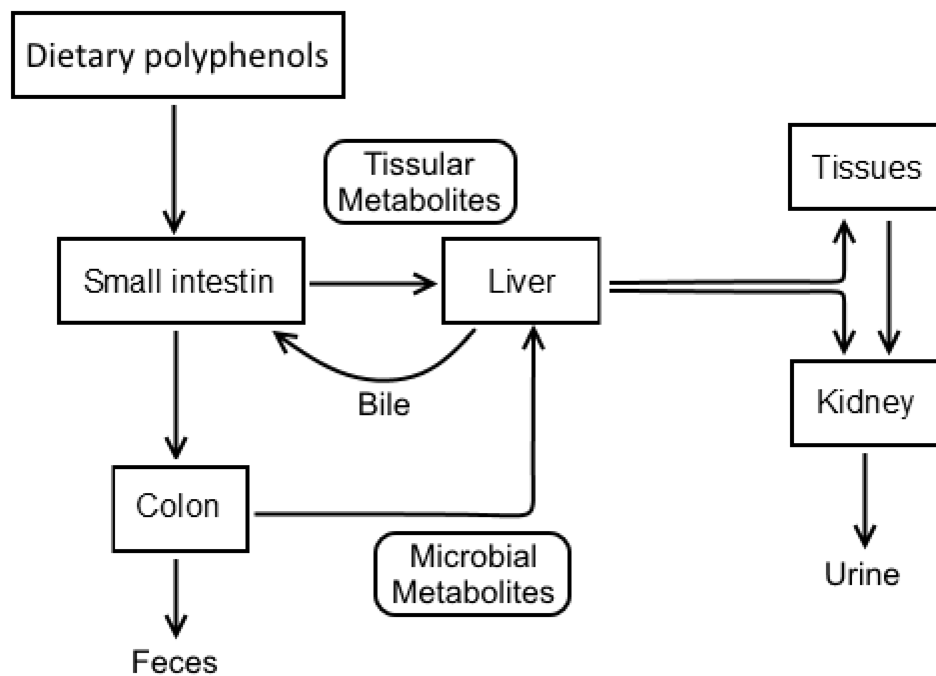


FIGURE 6 – Representation of the trajectory of polyphenols and metabolites in human's organism

Moreover, because the nature and the positions of the conjugating groups on the polyphenol structure may affect the biological properties, it is important that these circulating metabolites could be identified (Crozier, Rio, Del & Clifford, 2010; D'Archivio *et al.*, 2010). Evidence, although indirect, of their absorption through the gut barrier is given by the increase in the antioxidant capacity of the plasma after the consumption of polyphenols-rich foods (D'Archivio et al., 2007). However, tissue concentrations may be quite variable compared with plasma concentrations. As an example, isoflavonoid metabolites tend to accumulate in breast tissue while enterodiol and enterolactone in the prostate. This means that plasma polyphenols may not necessarily be the best biomarkers of exposure (Bohn, 2014). Therefore, the

determination of the bioavailability of the polyphenols in target tissues is much more important than the knowledge of their plasma concentrations. However, the investigation of the cellular and tissue uptake in humans is quite difficult, and consequently, the scarce information that exists in this field is derived mainly from animal studies whose extrapolation to humans needs to be demonstrated (Porrini & Riso, 2008).

Once ingested, a portion of low-molecular-weight polyphenols may be readily absorbed in the small intestine, while 90–95% accumulate in the large intestinal lumen, where they are subjected to the enzymatic activities of the gut microbial flora and transformed into a series of absorbable low-molecular-weight phenolic metabolites (Cardona *et al.*, 2013).

A diversity of colonic-derived catabolites is absorbed into the bloodstream and passes through the body prior to excretion in bile and/or urine. There is growing evidence that these compounds, which were little investigated until recently, are produced in quantity in the colon and form a key part of the bioavailability equation of dietary flavonoids and related phenolic compounds (Crozier, Rio, Del & Clifford, 2010).

1.2.3 Oxidative stress and inflammatory conditions

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, comonly known as reactive oxygen and nitrogen species (ROS and RNS), and their elimination by protective mechanisms, referred to as antioxidants. In biological systems, the most common source of reactive species is oxygen, mainly through mithocondria activity (Dröge, 2002). This organelle is responsible for the consumption of ~90% of cellular O_2 during the mitochondrial process, from which several short-lived intermediates are produced, including $O^{2\bullet\,-}$, H_2O_2 and $OH^{\bullet}$ (Khansari, Shakiba & Mahmoudi, 2009). Reactive species can also be produced by other celular enzymes such as xanthine oxidase (XOX) , nicotinamide adenine dinucleotide phosphate oxidase (NOX) and iNOS, which produces $NO^{\bullet}$ (Thatoi, Patra & Das, 2013).

To prevent the destructive effect of undesired oxidation reactions, cells make use of a plethora of antioxidant mechanisms consisting of several enzimatic (SOD, GSH-px, CAT, etc) and non-enzimatic (vitamine A, C, E) elements, allowing them to keep ROS and RNS in low concentrations (Santhakumar, Bulmer & Singh, 2014). When the homeostasis between antioxidants and reactive species is disrupted, overproduction of ROS and RNS becomes toxic to cells and tissues, reacting uncontrollably with endogenous macromolecules including lipids, proteins and DNA (Balamurugan & Karthikeyan, 2012). This self agression together with exogenous agressions (ultraviolet light, ionizing radiation, and pollutants, traumatisms, pathogens, etc) will cause the cells to enter in an oxidative-stress state.

Distinct oxidative-stress conditions are usually related with pathological events including those of inflammation (see fig. **7**).

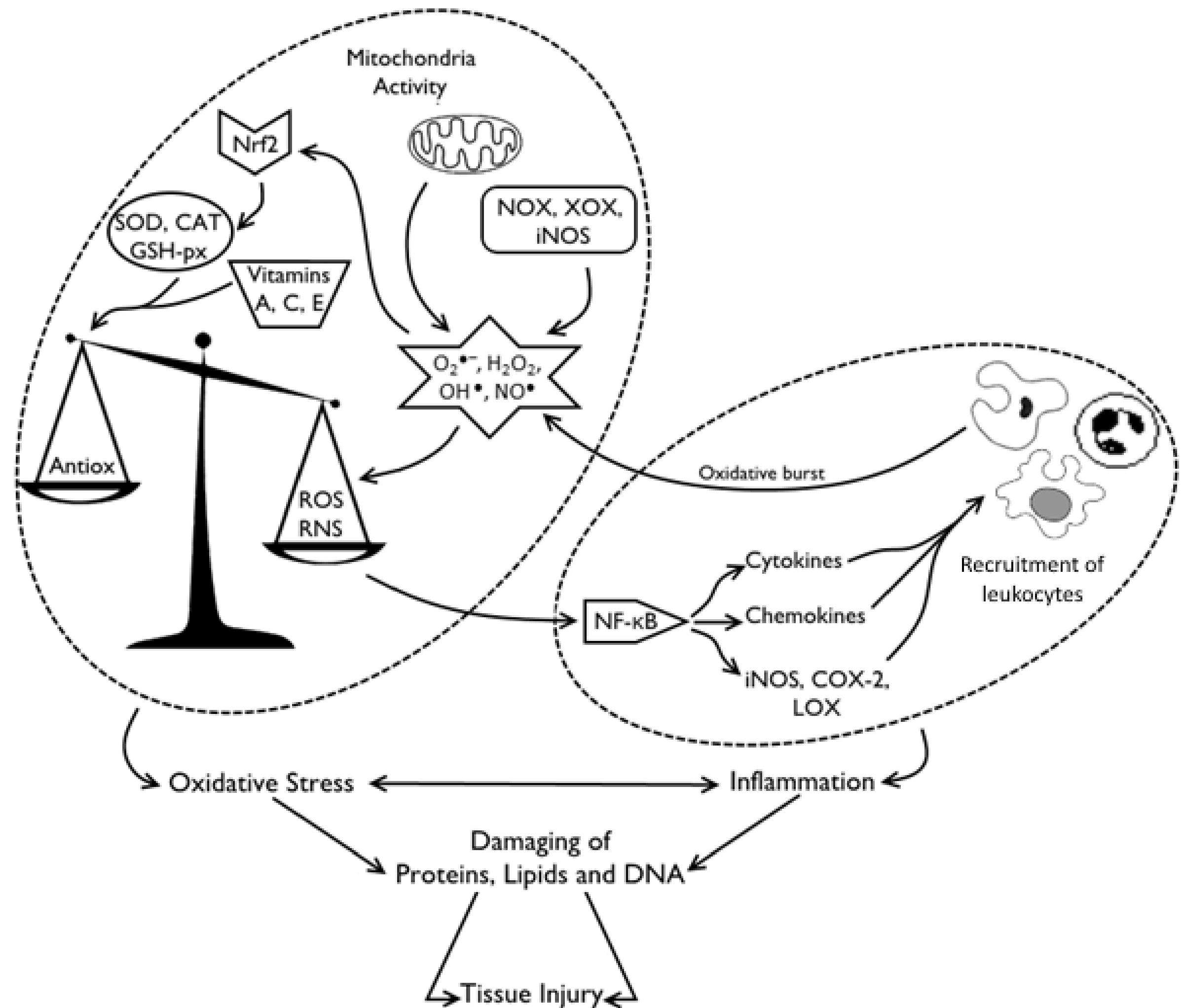


FIGURE 7 – Overview of the signaling cascades that mediate the oxidative stress and inflammatory conditions

Inflammation usually begins in a localized area, although depending on the injury´s severity, it can quickly become systemic. The acute phase of inflammation starts immediately upon injury and rapidly turns severe, persisting only for a short period of time (Meireles & Santos, 2010). Several chemical mediators, particularly cytokines and chemokines are released by phagocytic and nonphagocytic cells to stimulate the surrounding cells and recruit others from the immune system such as neutrophils and macrophages (Federico *et al.*, 2007). Some of the most well-known cytokines are the tumor necrosis factor-α (TNF-α), interleukin 1-β (IL1-β) and interferon-γ (IFN-γ). When these mediators are recognised, they will trigger a series of intracellular cascades that will promote the activation of the NF-κB, a crucial transcription factor closely related to inflammation that in turn increases the expression of genes coding pro-inflammatory cytokines, enzymes such as COX-2, LOX and iNOS, and also more ROS and RNS species (Cardoso et al., 2014). Notably, COX-2 and LOX are pivotal players in the arachidonic acid pathway, respectively controlling the biosynthesis of pro-

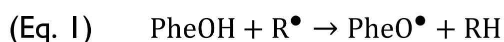
inflammatory eicosanoids and of leukotrienes (considered as potent mediators locally released at the inflammation site by leukocytes and other cells) (Vardeny & Solomon, 2008). The up-regulation of iNOS deeply increases the production of $NO^{\bullet}$ in cells, mainly in macrophages and endothelial cells. Together with other mediators, $NO^{\bullet}$ acts on the endotelial capillaries causing their dilatation, resulting in an increase of the blood flow on the affected area and making them more permeable to the blood proteins, neutrophils and macrophages which are being recruited so that they can move into the interstitial spaces. This results in an increase of the oxygen uptake leading to the oxidative burst, which is the second major endogenous source of reactive species (Reuter et al., 2010). Until they are contained, all these events result in a self-sustaining cycle where ROS and RNS stimulate the pro-inflammatory chemical mediators that in turn stimulate the production of more reactive species, establishing a perfect environment for the development of a chronic inflammation and several pathological conditions associated (Catarino et al., 2015).

1.2.3.1 Involvement of polyphenols in oxidative stress and inflammation

Three different molecular mechanisms are involved in the biological properties of polyphenols: I) neutralization of reactive species through direct scavenging of free radicals and chelation of heavy metals; II) inhibition of pro-oxidant, pro-inflammatory and redox-sensitive mediators such as NF-κB, iNOS, COX-2, LOX, XOX and NOX, and III) enhancement of cellular antioxidant defences through up-regulation of phase II detoxifying enzymes (González *et al.*, 2011; Santangelo *et al.*, 2007).

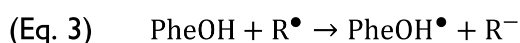
The first mechanism is related to the direct antioxidant effects of polyphenols and is the most well studied and clarified biological property of these compounds. Briefly, the phenolic compounds can accept electrons to form relatively stable phenoxyl radicals and this way, disrupting the chain oxidation reactions. This process may happen in two different ways, namely Hydrogen Atom Transfer (HAT) and by Single Electron Transfer (SET), which can be explained through the equations in below (Leopoldini *et al.*, 2004).

During the HAT reaction, a hydrogen atom is transferred from the phenolic compound (PheOH) to the radical ($R^{\bullet}$), resulting in a phenoxyl radical ($PheO^{\bullet}$) and a stable substance (RH) (Eq. 1). This phenoxyl radical can then react with other radicals or phenoxyl radicals (Eq. 2a and 2b, respectively) by radical-radical termination reactions, forming an unreactive compound, i.e., PheO–R or PheO–OPhe. On the other hand, in a SET reaction, the phenolic compound transfer one electron to reduce the radical, metals or carbonyls (Eq. 3) (Catarino *et al.*, 2015; Leopoldini *et al.*, 2004).



(Eq. 1) $\text{PheOH} + \text{R}^{\bullet} \rightarrow \text{PheO}^{\bullet} + \text{RH}$

(Eq. 2) $a)\ \text{PheO}^{\bullet} + \text{R}^{\bullet} \rightarrow \text{PheO} - \text{R}$ $\quad b)\ \text{PheO}^{\bullet} + \text{PheO}^{\bullet} \rightarrow \text{PheO} - \text{OPhe}$



(Eq. 3) $\text{PheOH} + \text{R}^{\bullet} \rightarrow \text{PheOH}^{\bullet} + \text{R}^{-}$

Chelation of transition metals such as Fe^{2+} is another perk of phenolic compounds, contributing this way to the reduction of the Fenton reaction, thus preventing oxidation caused by highly reactive hydroxyl radicals (Perron & Brumaghim, 2009).

Importantly, not all the existent polyphenols are good antioxidants. There are some structural aspects that grants some phenolic compounds better antioxidant activity than others. Regarding to the flavonoids, the higher number of hydroxyl groups, the better antioxidants they will be. However, the presence of the 3',4'-catechol and the 3-OH group are key features for their proper activity (Heim, Tagliaferro & Bobilya, 2002). The same rule of the hydroxyl numbers is applied to the phenolic acids, i.e., the higher number of OH groups, the stronger antioxidant power. Besides, if a methoxy group is present this power might even get enhanced. Moreover, comparing phenolic to hydroxycinnamic acids, the later possess significantly higher antioxidant abilities than the former, which means that the presence of the –CH=CH–COOH groups in hydroxycinnamic acids ensures greater H-donating ability and subsequent radical stabilization (Cai *et al.*, 2006).

Several chemical models have been developed in order to measure phenolics radical scavenging capacity. Although these methods reveal some limitations in terms of similarity to the mechanisms of antioxidant actions in a biological system, they still may portray well how polyphenols function as antioxidants, thus enlightening their role in human's health (Tsao, 2010).

Besides the direct antioxidant properties, polyphenols are also known to exhibit their abilities indirectly by acting on two fronts, i.e., by impairing the pro-oxidant and pro-inflammatory enzymes or ultimatly by enhancing the endogenous antioxidant defences (Higdon & Frei, 2003).

In this field, the most well clarified of structure-activity interactions have been described for flavonoids and XOX. According Cos *et al.* (1998) studies, the structural features that best adjust to a lower XOX activity are the presence of the C5 and C7 hydroxylations in the A-ring, as well as the presence of the C_2-C_3 doublebond in the C-ring.

Although less studied, some findings concerning the structure-activity relation of flavonoids and other enzymes have already been reported. According to the review of Kim *et al.* (2004), flavonols are more effective inhibitors of LOX, while flavones act preferentially against COX. Either way, evidences show that the presence of the same structural features

mentioned before, i.e., the C_2-C_3 double bond and the presence of the catechol, carbonyl and C5 and C7 hydroxyl groups, were also considered relevant for good inhibitory effect on these two enzymes as well as for the inhibition of iNOS, and IκB kinases which are responsible for the activation of the NF-κB transcription factor (Comalada *et al.*, 2006; Ribeiro *et al.*, 2014; Takano-Ishikawa, Goto & Yamaki, 2006).

Very limmited information exists regarding to the structure-activity between hydroxycinnamates and pro-oxidant and/or pro-inflammatory enzymes. However, some evidences have already demonstrated that the 3,4-catechol and the α,β-unsaturated groups are important for an effective inhibition of XOX activity and NF-κB biding to DNA (Chang *et al.*, 2007; Chiang *et al.*, 2005).

1.3 Eriocephalus africanus L. and Geranium robertianum L.

Eriocephalus africanus L. (Asteraceae) (Fig. **8** A) and *Geranium robertianum* L. (Geraniaceae) (Fig. **8** B) are traditionally used as medicinal plants, though *E. africanus* is more prevalent among the South African provinces while *G. robertianum* is more popular in European countries, with many therapeutic applications, including the treatment of inflammatory and oxidative stress-related diseases (Njenga & Viljoen, 2006; Tofts, 2004).

FIGURE 8 –*Eriocephalus africanus* L. (A) and *Gernaium robertianum* L. (B)
(A) Aparicio, Divina (2010) retrieved from: *http://www.biodiversidadvirtual.org/herbarium/Eriocephalus-africanus-3-4-img26641.htm;* (B) Goff, MrPany (2011) retrieved from: *https://commons.wikimedia.org/wiki/File:Geranium_robertianum_-_Etropole.jpg*

E. africanus, a plant native from South Africa and naturalized in the Mediterranean region (Fig. **9** A) where it is commonly known as "Rosmaninho Africano" (Portuguese), "Kapokbos" (Afrikaans), "Cape snowbush" or "Wild rosemary", is a small fast growing evergreen shrub, with green-grey foliage and snow white flowers of a distinctive fragrance that give rise to

cottony seeds (Merle *et al.*, 2007; Salie, Eagles & Leng, 1996). Frequently used for cooking, dried or fresh leaves and young tops of *E. africanus* are finely chopped and used in soups, salads, vegetables, meat and other foods. Besides, it can also be used to flavour wines, vinegar, oil and butter (Catarino *et al.*, 2015).

G. robertianum commonly known as "Erva de São Roberto" (Portuguese), "Herb-Robert" or "Red Robin" is an annual or biennial herbaceous plant native from Europe and temperate Asia, and introduced in North America (Fig. **9B**) (Svendsen *et al.*, 2015). It grows in moist places up to 50 cm high, with reddish stems and green leaves that also turn red at the end of the flowering season when it produces small and pink, five-petalled flowers (Tofts, 2004).

The Southern Africans have been known to use *E. africanus* for the treatment of gastro-intestinal disorders, asthma, coughs, fever and painful conditions and as diuretic, diaphoretic, antiseptic, anti-inflammatory and antioxidant (Njenga & Viljoen, 2006; Njenga, 2005; Salie, Eagles & Leng, 1996), while *G. robertianum* has been employed in the treatment of conjunctivitis, rashes, bruises, swellings, tooth-ache, fever, stomach and gut, afflictions of the kidneys and genitalia, and used as anti-inflammatory, anti-asthmatic, anti-allergic, antioxidant, antidiarrhoeic, antihepatotoxic, diuretic, tonic, haemostatic and antidiabetic (Jemia *et al.*, 2013; Tofts, 2004).

FIGURE 9 – Approach to the geographical distribution of *E. africanus* (A) and *G. robertianum* (B) around the world. This maps are based on occurrence records available through the GBIF Backbone Taxonomy, 2013-07-01 and may not represent the entire distribution. Accessed via http://www.gbif.org/species/2890668 on 2015-07-16

The Southern Africans have been known to use *E. africanus* for the treatment of gastro-intestinal disorders, asthma, coughs, fever and painful conditions and as diuretic, diaphoretic, antiseptic, anti-inflammatory and antioxidant (Njenga & Viljoen, 2006; Njenga, 2005; Salie, Eagles & Leng, 1996), while *G. robertianum* has been employed in the treatment of conjunctivitis, rashes, bruises, swellings, tooth-ache, fever, stomach and gut, afflictions of the kidneys and

genitalia, and used as anti-inflammatory, anti-asthmatic, anti-allergic, antioxidant, antidiarrhoeic, antihepatotoxic, diuretic, tonic, haemostatic and antidiabetic (Jemia *et al.*, 2013; Tofts, 2004).

Despite all this popular knowledge, scientific data supporting these health benefits is still scarce, and even more concerning the constituents that may be responsible for them. This is particularly true for *E. africanus* since only few publications can be found indexed to the ISI Web of Science® platform (Fig. **10** A).

Notwithstanding, there are already a few records supporting some of the beneficial properties of *E. africanus* and *G. robertianum* as well. In particular, organic (ethanol, methanol and chloroform) extracts of *E. africanus* have been shown to exhibit antifungal capacity against *Candida albicans*, while organic and aqueous extracts were reported to be effective against *Staphylococcus aureus* (Salie, Eagles & Leng, 1996). Also, an aqueous extract of *E. africanus* has been proved to significantly attenuate nociception and pyrexia in *in vivo* mice models, thus confirming its analgesic and antipyretic properties (Amabeoku et al., 2000). Moreover, antipyretic, analgesic, anti-allergic, anti-depressant, antiseptic, anti-inflammatory and antioxidant properties were reported for acetone extracts and essential oils of *E. africanus* leaves.

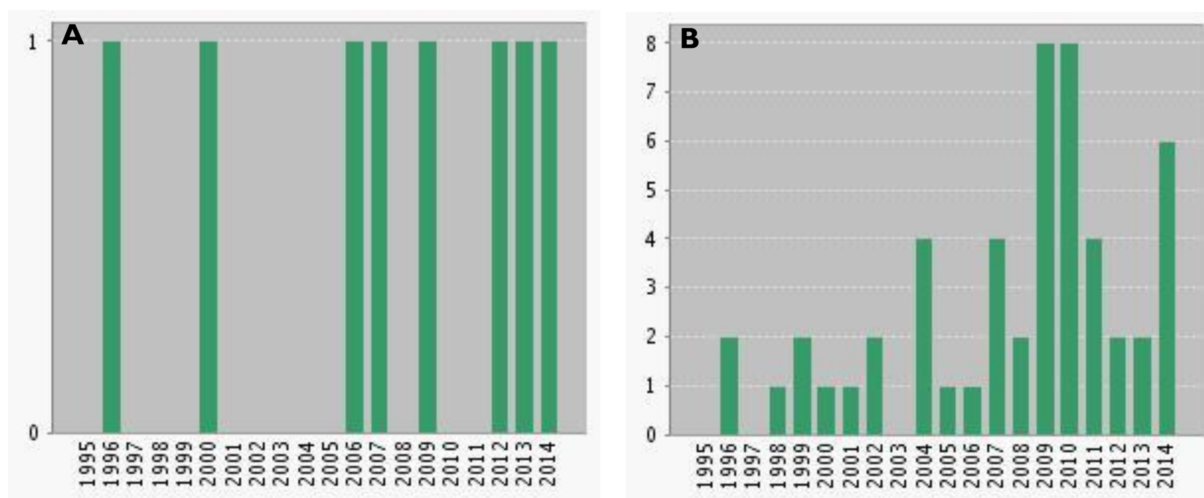


FIGURE 10 – Number of publications in the 1995 - 2014 period by simple search in ISI Web of Science® using the keywords "*Eriocephalus africanus*" (A) and “*Geranium robertianum*” (B)

Likewise, different extracts (water, aqueous ethanol and methanol) as well as polyphenolic purified and/or concentrated extracts of *G. robertianum*, were already reported for their potential antioxidant activity upon assayed in several chemical methods including DPPH•, ABTS•+, HOCl, reducing power and metal chelating activity (Amaral *et al.*, 2009; Fodorea *et al.*, 2005; Jemia *et al.*, 2013; Kobakhidze & Alaniya, 2004; Neagu *et al.*, 2010; Roman *et al.*, 2009). Furthermore, leaf decoctions of this plant were shown to decrease the plasma glucose levels in diabetic rats and improve liver mitochondrial respiratory parameters, increasing the oxidative phosphorylation efficiency, which can explain a possible antidiabetic mechanism of this

plant (Ferreira *et al.*, 2010). Additionally, Paun *et al.* (2013) demonstrated that *G. robertianum* could be considered as possible remedy in ulcer treatment since the aqueous phenolic extracts of this plant were very effective inhibitors of urease, an enzyme that is closely associated to the pathogenesis of gastric and peptic ulcers. Interestingly, due to its abundance in ellagitanins, *G. robertianum* may contribute as a substrate for the production of urolithins metabolites (resultant from the metabolism of ellagic acid and its derivatives by the gut microbiota) (Piwowarski *et al.*, 2014). Since evidence suggest that urolithins are correlated with anticarcinogenic, anti-inflammatory, antioxidant, antimicrobial and other health promoting properties (Espín *et al.*, 2013), it is possible to assume that *G. robertianum* possess this same features in an indirectly manner.

Limited information regarding to the phenolic composition of these plants is known as well. As a matter of fact, to the author's knowledge, *E. africanus* phenolic profile has never been described before. On the other hand, records of the phenolic composition of *G. robertianum* determined by HPLC have already been reported before. However since in that work the extracts were all submitted to an hydrolysis step, only the aglycones have been identified (Fodorea *et al.*, 2005).

OBJECTIVES

2 OBJECTIVES

Eriocephalus africanus and *Geranium robertianum* are two different plant species that are known in folk medicine for their health benefits. Despite the scarce scientific information, some studies have already reported their therapeutical potential in the treatment of some pathological conditions, suggesting that these plants could be a source of biological active compounds, such as polyphenols, which could be used for medicinal purposes.

In this context, the research work present in this master thesis intended to investigate the cytotoxicity, antioxidant and anti-inflammatory potential of these two plant species with association to their phenolic profile in order to better understand the involvement of their phenolic constituents on their beneficial properties. The final purpose is to explore the potential of *Eriocephalus africanus* and *Geranium robertianum* for being economically exploited as therapeutic and/or nutraceutical agents for the treatment and/or prevention and/or attenuation of the symptoms associated to oxidative stress-related pathological conditions.

To accomplish this goal, the following tasks were proposed:

- Determination of antioxidant activities of *Eriocephalus africanus* and *Geranium robertianum* hydroethanolic extracts
- Evaluation of the anti-inflammatory properties and cytotoxicity of *Eriocephalus africanus* and *Geranium robertianum* hydroethanolic extracts
- Characterization of the phenolic constituents of *Eriocephalus africanus* and *Geranium Robertianum* hydroethanolic extracts by high performance liquid chromatography associated with diode array detection and electrospray mass spectrometer (HPLC-DAD-ESI-MS^n)

EXPERIMENTAL PROCEDURE

3 EXPERIMENTAL PROCEDURE

3.1 Chemicals

The phenolic standards chlorogenic acid, ferulic acid and eriodictyol were obtained from Extrasynthese (Genay Cedex, France). Ethanol, methanol, *n*-hexane and acetonitrile of HPLC purity were purchased from Lab-Scan (Lisbon, Portugal). PBS reagents (sodium salt, sodium chloride, potassium chloride, disodium hydrogenophosphate and potassium dihydrogenophosphate), iron(II) sulphate, potassium hexacyanoferrate(III), iron chloride(III), ABTS diammonium salt, gallic acid, trolox, BHT, TCA, DPPH, DMEM, Tween 20®, penicillin G sodium salt, streptomycin sulphate salt, sodium bicarbonate, LPS from *E. coli* – serotype 026:B6, linoleic acid and soybean 5-LOX were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid, formic acid, Folin–Ciocalteu reagent, sodium carbonate, sodium phosphate, potassium hydroxide and EDTA were purchased from Panreac (Barcelona, Spain). Fluorescein disodium salt, AAPH and TBA were purchased from Acros Organics (Geel, Belgium), while FBS and trypsin from Gibco (Paisley, UK). Hydrogen peroxide and sodium hydroxide were purchased from Fisher Scientific (Hampton, USA). Boric acid was purchase from Chem-lab (Zedelgem, Belgium), deoxyribose from Alfa Aesar (Massachusetts, USA) and mannitol from MERCK (New Jersey, USA)

3.2 Plant material

The plants of *E. africanus* were cultivated and collected from the fields of the Coimbra College of Agriculture, while the *G. robertianum* specimens were collected from the spontaneous growing from Cernache do Bonjardim and botanically identified by Professor Hélia Marchante from Coimbra College of Agriculture / Center of Functional Ecology of the University of Coimbra. After collection, leaves were separated from the stems and the two parts of the plants were separately dried for 5 days at 36 °C in a ventilated incubator.

3.3 Extraction of phenolic compounds

Dried stems and leaves (10 g) of *E. africanus* and *G. robertianum* were separately grounded in a sieve with 0.5 mm, and extracted with 150 mL of an 80% ethanol solution (v/v) at room temperature for 20 min and the resulting solutions were filtered through G4 sintered plates filter. The residues were re-extracted in the same conditions for a total of three times and the filtrated solutions were concentrated under reduced pressure in a rotary evaporator at 37 °C, following defatting with equal volume of *n*-hexane. The defatted solutions were then

frozen at 20 °C and freeze-dried. The resultant dried material from stem and leaves of *G. robertianum* (GRS and GRL, respectively) or *E. africanus* (EAS and EAL, respectively) hydroethanolic extracts were then stored in vacuum in a desiccator in the dark, for subsequent use.

3.4 Antioxidant properties

3.4.1 DPPH• scavenging assay

This method consists in the color change of DPPH from purple (DPPH•) to yellow (DPPH-H) in presence of a hydrogen donner substance. This assay was performed following the procedure previously described (Catarino *et al.*, 2015). Extract solutions were prepared with six different concentrations ranging from 0.1–1 mg/mL for *E. africanus* and 0.05 – 0.5 mg/mL for *G. robertianum*. From these solutions, 0.1 mL were added to 1.9 mL of a 76 µM methanolic solution of DPPH• to complete a final volume of 2 mL. After 30 min of incubation in the dark, the absorbance of the mixtures was measured in a spectrophotometer (Dr Lange, XION 500, Germany) at 517 nm, against a blank (absence of DPPH•). The radical scavenging activity of each ethanolic extract was calculated as the percentage of DPPH• discoloration, using the equation of Yen e Duh (1994):

(Eq. 4) $$\text{Inhibitory}\ \% = \frac{Ac_0 - Ae_t}{Ac_0} \times 100\ ,$$

where Ac_0 is the absorbance of the solution without adding sample, and Ae_t is the absorbance of the solutions with the extracts, read at the end of the reaction. Based on chart values of percentage of DPPH• inhibition vs. extract concentration, the IC_{50} (concentration of the extract able to inhibit 50% of the DPPH•) of each extract was estimated. Each experiment was carried out at least three times with triplicates, and ascorbic acid was used as standard compound (2.8 – 28.0 µM).

3.4.2 $ABTS^{\bullet+}$ decolorization assay

The mechanistic basis of this assay is pretty close to that of DPPH• assay, i.e., in presence of a hydrogen donner substance, the dark green color fades as the ABTS radical cation gets reduced. This method was performed according to the described by Yang *et al.* (2013) with some modifications. A stock solution of $ABTS^{\bullet+}$ was prepared by reacting the $ABTS\text{-}NH_4$ aqueous solution (7 mM) with 2.45 mM potassium persulfate (final concentration) and stored in the dark at room temperature for 12–16 h to allow the completion of radical generation. This solution was then diluted with ethanol so that its absorbance was adjusted to 0.70 ± 0.02 at 734 nm. To determine the scavenging activity, solutions of concentrations ranging 0.06 – 1

mg/mL of *E. africanus* and 0.13 – 1 mg/mL of *G. robertianum* extracts were prepared, and 100 µL of each were mixed with 1 mL of diluted $ABTS^{\bullet+}$ completing a final volume of 1.1 mL. Absorbance was then measured at 734 nm in a spectrophotometer (Dr Lange, XION 500, Germany) against ethanol (used as blank) after 20 min of incubation in the dark at room temperature. The percentage of inhibition of $ABTS^{\bullet+}$, was again calculated using Eq. 4 (see **3.4.1**) and the IC_{50} values were determined as well by plotting the percentage of $ABTS^{\bullet+}$ inhibition against extracts concentration. Three repetitions were carried out for each experiment and ascorbic acid 2.4 – 38.8 µM which was used as standard compound.

3.4.3 $OH^{\bullet}$ scavenging assay

In this assay the hydroxyl radicals generated by ferric-ascorbate-EDTA-H_2O_2 will attack deoxyribose to form products called thiobarbituric acid reactive substances (TBARS), which upon heating with TBA at low pH yield a pink chromogen. When a hydroxyl scavenger substance is added to the reaction, it will compete with deoxyribose for hydroxyl radicals decreasing the TBARS formation and consequently the pink coloration. This assay was conducted following the procedure described by Kumar *et al.* (2006). A solution (140 µL) composed of $FeCl_3$ (75 µM), EDTA (300 µM) and H_2O_2 (8.4 mM) prepared in 17.14 mM phosphate buffer (pH=7.4), was mixed with 210 µL of extract solutions at different concentrations (56 – 336 µg/mL), 35 µL of deoxyribose (33.6 mM) and 35 µL of ascorbate (1.2 mM). The reaction mixture was then incubated at 37 °C over 60 min to allow the generation of hydroxyl radicals by ferric-ascorbate-EDTA-H_2O_2 interactions. Afterwards, 350 µL of 1% (w/v) TBA (prepared in 50 mM of NaOH) and equal volume of 5% (w/v) TCA were added and the solutions were placed in a boiling water bath for 15 min, to allow the formation of the pink chromogen. The reactions were then interrupted in an ice bath and the absorbance was measured in the spectrophotometer at 532 nm. The percentage of the inhibition of this reaction by the scavenging of $OH^{\bullet}$ was calculated according to the Eq. 4 (see **3.4.1**), and the IC_{50} determined through linear regression by plotting the percentage of inhibition against concentration of the extracts. Three experiments for each sample were performed in triplicate and mannitol (0.3 – 10.3 µM) was used as reference compound.

1.2.1 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay is a simple method based on the ability of a substance to reduce the iron from its ferric form (Fe^{3+}) to the ferrous form (Fe^{2+}) and was performed according to the

procedure described before (Catarino *et al.*, 2015). For each extract, solutions of eight different concentrations (0.01–0.09 mg/mL) were prepared, and 0.5 mL of each was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium hexacyanoferrate aqueous solutions. After 20 min of incubation at 50 °C, 2.5 mL of 4% (w/v) TCA was added followed by a vigorous stirring. Thereafter, 2.5 mL of each solution was transferred to new vials where 2.5 mL of deionized water and 0.5 mL of 0.1% (w/v) of iron chloride(III) were added, and the absorbance was then measured at 700 nm. A linear regression analysis was carried out by plotting the mean absorbance against concentrations, and the IC_{50} value was determined considering the extract concentration that provides 0.5 of absorbance. All the experiments were performed at least three times in triplicate and BHT (8.1 – 33.2 μg/mL) was used as a reference compound.

3.4.4 Lipid peroxidation inhibitory capacity in the presence of thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation refers to the oxidative degradation caused by the attack of free radicals to lipids in cell membranes and causing a chain reaction. The resultant end products are reactive aldehydes such has malondialdehyde (MDA) which in the same conditions referred in the **3.4.3** is also a TBARS. The presence of the antioxidant substances will interrupt the reaction propagation thus decreasing the MDA formation and the pink coloration. Following the method described by Catarino *et al.* (2015), 100 μL of 1.4% (w/v) swine brain homogenate was incubated with solutions of five different concentrations (0.1–0.5 mg/mL for *E. africanus* extracts and 0.04 – 0.2 mg/mL for *G. robertianum* extracts) and the lipid peroxidation was initiated by the addition of 100 μL of iron sulfate(II) (10 mM) plus 100 μL of ascorbic acid (0.1 mM). After incubation at 37 °C for 30 min, 500 μL of 28% (w/v) TCA and 380 μL of 2% (w/v) TBA were added to this reaction mixture which was again incubated in a boiling water bath for 20 min. The tubes were then centrifuged at 3500 rpm for 10 min at the temperature of 4 °C. The extent of lipid peroxidation was evaluated on the supernatant by the estimation of TBARS level by measuring the absorbance at 532 nm. The inhibitory percentage for each concentration used was then calculated using Eq. 4 (see **3.4.1**), and through linear regression analysis the IC_{50} was calculated considering the concentration of sample that caused 50% reduction of MDA formation. Trolox (0.04 – 0.2 μM) was used as the reference compound.

3.4.5 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed according to the method described by Rashidinejad *et al.*, (2013) with some modifications. In a 96-well, 150 µL of fluorescein (10 nM), prepared from a stock solution of 250 µM by diluting in 75 mM phosphate buffer (pH 7.4), were placed together with 25 µL of trolox standards (3.13 – 25 µM) and samples with final concentrations ranging between 1.25 – 6.25 µg/mL (stems extract of *E. africanus*) or 1.25 – 12.5 µg/mL (remaining samples). For blanks, 25 µL of phosphate buffer were added instead of antioxidant solutions. After an incubation of 10 min at 37° C, 25 µL of AAPH (153 mM) solution was added to each well, to a final reaction volume of 200 µL. The plate was immediately placed in the plate reader (SLT, Austria) and fluorescence was monitored every minute over 60 min. The measurement was carried out at 37 °C with automatic agitation for 5 secs prior to each reading. Excitation was conducted at 485 nm with a 20 nm bandpass and emission was measured at 528 nm with a 20 nm bandpass.

Six concentration dependent kinetic curves were obtained for each sample, as well for trolox (standard compound, see curves in Fig. **11)**.

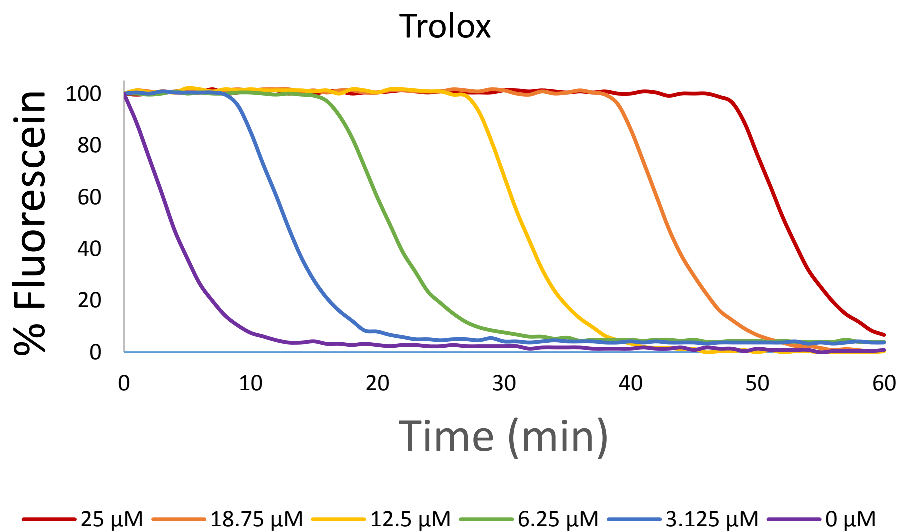


FIGURE 11 – Plots of Trolox Kinetic Curves. Representative curves from ORAC assay of varying concentrations of Trolox antioxidant standards ranging from 0 to 25 µM.

The area under the curve (AUC) of the fluorescence decay and Net AUC were calculated according to the following equations:

(Eq. 5) $$AUC = 1 + \sum_{t_0=60\,min}^{t_i=60\,min} \frac{R_i}{R_0},$$

(Eq. 6) $$Net\ AUC = AUC_{Sample} - AUC_{Blank},$$

where R_I is the fluorescence reading at the initiation of the reaction and R_i was the fluorescence read at the time i.

Linear regression analysis by plotting the Net AUC values against their correspondent concentration, allowed to obtain the slope (m) of the curve equations for each sample and standard. Antioxidant activities (ORAC value) were calculated by the following ratio:

(Eq. 7) $$TE = \frac{m_{Sample}}{m_{Trolox}},$$

All the experiments were conducted at least three times in triplicate, and the final results were expressed in μM of Trolox equivalent/μg (TE μM/μg) of sample extract.

3.4.6 *Chemical NO• scavenging assay*

Chemical NO• scavenging is a method possessing two valences, i.e., similarly to the scavenging assays mentioned before, it allows to evaluate the antioxidant potential of the samples in study by testing their ability to arrest this radical, but since NO• is also a very important mediator in inflammation, this method also allows a preliminary screening of the samples with anti-inflammatory potential.

The scavenging effects of *E. africanus* (83.33 – 666.67 mg/mL) and *G. robertianum* (3.26 – 26.04 mg/mL) extracts on NO• scavenging ability were evaluated according to the method described by (Bor, Chen & Yen, 2006). Incubation of 200 μL of sodium nitroprusside (3.33 mM) in phosphate buffer saline 100 mM (pH=7.4) with or without 200 μL of the different sample concentrations was performed at room temperature under light irradiation for 10 min. The NO• thus generated interacts with molecular oxygen, producing NO_2^- which in presence of 200 μL of *Griess* reagent (1% of sulfanilamide and 0.1% of naphthylethylenediamine dihydrochloride in 2.5% of phosphoric acid) produces a purple azo dye caused by the diazotization with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride. The measurement of the absorbance was determined spectrophotometrically at 562 nm (Dr Lange, XION 500, Germany), and the IC_{50} value for the NO• scavenging activity was determined by plotting the percentage of inhibition of nitrite generation in the presence of the plant extracts (also calculated through Eq. 4 mentioned in **3.4.1**) against the tested concentrations. Each experiment was conducted at least three times with triplicates and ascorbic acid (3.3 – 55 μg/mL) was used as standard compound.

3.5 *Evaluation of the hepatocytes viability*

3.5.1 *Cell culture*

Human hepatic carcinoma cell line (HepG-2—ATCC HB-8065) was grown in DMEM supplemented with 1 g/L of glucose and pyruvate, 10% (v/v) inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/ml streptomycin and 1.5 g/L of sodium bicarbonate, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Along the experiments, cells were monitored by microscopic observation in order to detect any morphological change.

3.5.2 *Hepatocytes viability by the MTT assay*

Liver is the most important detoxifying organ in human body, thus it is important to evaluate the potential cytotoxic effects that the studied extracts might have over the hepatic cells. Therefore, the assessment of the hepatocytes viability was performed using MTT reduction colorimetric assay, as previously reported (Bufalo *et al.*, 2013). This method relies on the metabolization of the yellow tetrazolium salt into the insoluble purple formazan inside living cells. A solution of acidic isopropanol is then added to solubilize the formazan allowing to read and quantify the absorbance spectrophotometrically. Thus, HepG-2 cells (0.08×10^6 cells/well in 96-well culture plates) were plated and allowed to stabilize overnight. Afterwards, cells were either maintained in culture medium (control) or incubated for 24h with different concentrations of previously filtered (0.2 µm PVDF whatman syringe filter, GE Healthcare Life Sciences, UK) EAS extract (final concentrations 50 – 200 µg/mL) and both *G. robertianum* extracts (25 – 100 µg/mL). After treatments, a solution of MTT (5 mg/mL in PBS) was added to cells, which were further incubated at 37 °C for 60 min. Finally, culture supernatants were removed and dark blue crystals of formazan were solubilized with 300 µL acid isopropanol (0.04N HCl in isopropanol). Quantification of formazan was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

3.6 *Anti-inflammatory properties*

3.6.1 *Cell culture*

The murine macrophage cell line RAW 264.7 (ATCC number: TIB-71) was cultured in DMEM supplemented with 10% (v/v) non-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Along the experiments, cells were monitored by microscopic observation in order to detect any morphological change.

3.6.2 Cell viability by MTT assay

Assessment of metabolically active cells was also performed using MTT reduction colorimetric assay. RAW 264.7 cells (0.3×10^6 cells/well in 48-well culture plates) were plated and allowed to stabilize overnight. Afterwards, cells were either maintained in culture medium (control) or incubated for 1h with different concentrations of previously filtered (0.2 μm PVDF whatman syringe filter, GE Healthcare Life Sciences, UK) EAS extract (final concentrations 50 – 200 μg/mL) and both *G. robertianum* extracts (25 – 100 μg/mL). Macrophages were later activated with 1 μg/mL LPS for 24 h. After treatments, a solution of MTT (5 mg/mL in PBS) was added to cells, which were incubated at 37 °C for 15 min, and further processed as described in the section **3.5**.

3.6.3 Inhibition of cellular NO• production

As referred before, NO• is a very important mediator overproduced during the inflammatory response. Therefore, the reduction of this pro-inflammatory molecule would be beneficial for alleviation of an inflammatory status. Thus, the anti-inflammatory potential of both extracts was evaluated using an *in vitro* model of inflammation, the lipopolysaccharide (LPS)-stimulated macrophages cell line RAW 264.7, as described by (Bufalo *et al.*, 2013). The accumulation of nitrites in the culture supernatants as the result of NO• production by macrophages stimulated with LPS was measured using a colorimetric reaction with the *Griess* reagent. Cells were plated at a density of 0.3×10^6 cells/well in 48-well culture plates, allowed to stabilize overnight, and then incubated with culture medium (control), or pre-stimulated with 1 μg/mL LPS during 60 min in the absence or in the presence of four concentrations of previously filtered (0.2 μm PVDF Whatman syringe filter, GE Healthcare Life Sciences, UK) stems extract of *E. africanus* and both extracts from *G. robertianum* (final concentrations 50 – 200 μg/mL and 25 – 100 μg/mL, respectively), for 24 h. Briefly, 170 μl of culture supernatants were collected and mixed with an equal volume of the *Griess* reagent during 30 min, in the dark. The absorbance at 550 nm was measured using an automatic plate reader (SLT, Austria). Nitrite concentration was determined through interpolation of the values using a sodium nitrite standard curve using concentrations ranging from 0.25 – 250 μM. This experiment was carried out three times in triplicate for each tested sample.

3.6.4 Soybean 5-Lipoxygenase (5-LOX) assay

5-Lipoxigenase is an enzyme known to catalyze the oxidation of unsaturated fatty acids containing 1 – 4 pentadiene structures such as linoleic acid which, in accordance with previous studies (Tappel, Lundberg & Boyer, 1953), was used in this assay as the substrate. Linoleic acid (250 μM) was prepared by dilution of a stock solution (1M) in 0.2 M borate buffer (pH=8.0) containing 0.05% (v/v) Tween® 20. In a quartz cell, a mixture of 1.94 mL of linoleic acid with 30 μL of each sample (5 mg/mL) was prepared and the reaction was initiated by the addition of 60 units of soybean 5-LOX in a final solution of 2 mL. The cell was immediately placed in an UV/vis spectrophotometer (UVmini-1240 UV-VIS Spectrophotometer, SHIMADZU, Japan) and the absorbance was recorded every 30 sec over 5 min, at 234 nm. As the linoleic acid is converted to 1–3-hydroperoxy linoleic acid, the appearance of a conjugated diene promotes the increase in the absorbance that is proportional to the reaction time generating a curve. The value for % inhibition of enzyme activity was calculated as follows

(Eq. 8) $$\text{Inhibitory}\ \% = \frac{m_{Ac0} - m_{Aet}}{m_{Aet}} \times 100,$$

where m_{Ac0} is the slope of the straight line portion of the curve generated by the negative control and m_{Aet} the slope of the straight line portion of the curve generated by each sample. Three repetitions of this experiment were carried out for each sample and for ascorbic acid (75 μg/mL), which was used as standard compound.

3.6.5 Cell lysates and Western blot analysis for measurement of iNOS and COX-2 levels

The aim of this assay was to evaluate whether the extracts studied could interfere with the protein levels of iNOS and COX-2, which are key players overexpressed in inflammatory conditions. For that, RAW 264.7 cells treated as previously described (see **3.6.3**) were pre-incubated during 1 h with or without 100 μg/mL (final concentration) of *G. robertianum* stems extract prior to the stimulation of 1 μg/mL of LPS, or just culture medium (for control) and further incubated for 24 h. Afterwards, cells were washed with ice-cold PBS, and incubated with RIPA lysis buffer (50 mM Tris–HCl, pH 8.0; 1% (v/v) Nonidet P-40; 150 mM NaCl; 0.5% (w/v) sodium deoxycholate; 0.1% (w/v) SDS; 2 mM EDTA), freshly supplemented with protease and phosphatase inhibitors cocktail, and sonicated for 7 sec at 40 μm peak to peak in Vibra Cell sonicator (Sonics & Material INC.) to decrease viscosity. The nuclei and the insoluble cell debris were then removed by centrifugation (12,000 g for 10 min at 4 °C), and the postnuclear extracts were collected and used as total cell lysates. Protein concentration was achieved using the bicinchoninic acid method described by (Smith *et al.*, 1985) before denaturation of lysates

at 95 °C, for 5 min, in sample buffer (0.125 mM Tris, pH 6.8; 2% (w/v) SDS; 100 mM DTT; 10% (v/v) glycerol and bromophenol blue). Western blot analysis was subsequently performed as previously described (Bufalo *et al.*, 2013), for evaluation of protein levels of iNOS and COX-2. Briefly, 30 µg of protein were electrophoretically separated on a 10% (v/v) sodium dodecyl sulfate polyacrylamide gel and transferred to a PVDF membrane. The membranes were blocked with 5% (w/v) fat-free dry milk in TBS-T, for 1h, at room temperature. Blots were then incubated overnight at 4 °C with the primary anti-iNOS (1:1000) mouse monoclonal antibody, and anti-COX-2 (1:10,000) rabbit polyclonal antibody. After washing the membranes for 3 times during 10 min with TBS-T, these were further incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-mouse and anti-rabbit antibodies (for iNOS and COX-2, respectively). The detection of the immune complexes was followed by scanning for blue excited fluorescence on the Typhoon imager (GE Healthcare) after 5 min of membrane exposure to the ECF reagent. The generated signals were analyzed using the ImageQuant TL software. Thereafter, membranes were stripped and the same process was repeated for α-tubulin which was used as loading control and for the normalization of the results.

3.7 Quantification and identification of phenolic compounds through HPLC–DAD–ESI/MS^n

The total phenolic content of each extract was quantified through Folin – Ciocalteu method (Pereira *et al.*, 2012) with some modifications. A mixture of 1580 µL of milliQ water, 100 µL of Folin–Ciocalteu reagent and 20 µL of plant extract solutions (2 mg/mL) were prepared. After 3 min, 300 µL of sodium carbonate 17% (w/v) were added and the mixture was homogenized and incubated for 30 min at 40 °C. The absorbance was measured at 700 nm and the amount of total phenolic compounds was expressed as gallic acid equivalent (mg GAE)/g dried weight of plant material, using a calibration curve of gallic acid as standard (0.001–0.01 µg/ml).

The LC-DAD-ESI/MS^n analysis was performed on an Ultimate 3000 (Dionex Co., California, USA) apparatus equipped with an ultimate 3000 Diode Array Detector (Dionex Co., California, USA) and coupled to a mass spectrometer.

The chromatographic system consisted of a quaternary pump, an autosampler, a degasser, a photodiode-array detector and an automatic thermostatic column compartment. Analysis was run on a Hichrom (Theale, UK) Nucleosil C18 column (250 mm length; 4.6 mm i.d.; 5 µm particle diameter, end-capped) and its temperature was maintained at 30 °C. The mobile phase for the separation of *E. africanus* extracts constituents was composed of (A) acetonitrile

and (B) 0.1% of formic acid (v/v), both degassed and filtered before use. The solvent gradient started with 90–70% of solvent B over 20 min, from 70–40% of solvent B over 10 min, and from 40–0% of solvent B over 5 min following the return to the initial conditions. For the separation of the constituents of *G. robertianum* extracts, acetonitrile was replaced by (D) methanol degassed and filtered before use as well, and the solvent gradient applied started with 20–60% of solvent (D) over 40 min and from 60–100% over 5 min again returning to the initial conditions.

Prior to HPLC analysis, each extract (5 mg) was dissolved in 1 mL of 50% (v/v) of ethanol (v/v) and filtered through a 0.2 µm Nylon Whatman syringe filter (GE Healthcare Life Sciences, UK). The flow rate was 0.7 $mL.min^{-1}$ and split out 200 $\mu L.min^{-1}$ to MS. UV–Vis spectral data for all peaks were accumulated in the range 250–500 nm and chromatographic profiles were recorded at 280 nm.

The mass spectrometer used was an Amazon SL (Bruker Daltonics, Bremen, Germany) ion trap MS equipped with an ESI source. Control and data acquisition were carried out with the Compass Data Analysis data system (Bruker Daltonics, Bremen, Germany). Nitrogen above 99% purity was used and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 200 °C. The full scan covered the mass range from *m*/*z* 70 to 700. CID–MS/MS and MS^n experiments were simultaneously acquired for precursor ions using helium as the collision gas with collision energy of 10–40 arbitrary units.

The identification of individual phenolic compounds from *E. africanus* extracts was achieved by comparison of their retention times, UV–Vis spectra and MS^n spectra data with those of the closest available reference standards and data reported in the literature. The identification of those compounds for which no standard compounds were available was achieved only by comparison of the data reported in literature.

The quantification of the main individual phenolic compounds was only achieved by *E. africanus*, and was performed by peak integration at 280 nm, through the external standard method, using the most close reference compounds available. The detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves represented in table **2**, being defined as 3.3 and 10 times the value of the regression error divided by the slope, respectively (Ermer, Miller & Ermer Joachim, 2005; Snyder, Kirkland & Dolan, 2009). Note that phenolic compounds that were detected in MS analysis as a minor $[M-H]^-$ ion and were not simultaneously detected by UV-spectra analysis, were herein considered as trace components.

TABLE 2 – Linearity, LOD and LOQ of four standard compounds used as reference

Standard compound	Range concentration (µg/mL)	n[a]	Slope (area counts/mg)	Intercept (area counts/mg)	R^2	LOD (µg/mL)	LOQ (µg/mL)
3-CQA	31.3-500	5	666.8 (±22.6)	-7.1 (±4.1)	0.9996	20.6	62.5
FA	8.8-175	5	1232.4 (±48.2)	1.2 (±1.7)	0.9993	9.6	29.0
PCA	12.5-250	5	1366.5 (±28.4)	6.2 (±3.1)	0.997	18.4	55.7
ERD	3.3-30	5	983.5 (±30.2)	0.9 (±0.1)	0.9978	2.6	7.9

3-CQA, 3-caffeoylquinic acid; FA, ferulic acid; PCA, protocatechuic acid; ERD, eriodictyol

[a] Number of points used for the regression of standard solutions. Injections were done in triplicate

3.8 Statistical analysis

All the data were expressed as mean ± standard error of the mean (SEM) of three independent assays performed in duplicate and analyzed through ANOVA combined with Tukey's test (GraphPad Prism 5). The significance level was $p < 0.05$.

RESULTS

4 RESULTS

4.1 *Determination of the antioxidant activities*

4.1.1 *DPPH•, ABTS•+ and HO• scavenging activities*

Through these three assays it is possible to evaluate the antioxidant potential of the plant extracts by measuring their ability to scavenge the DPPH, $ABTS^+$ and OH radicals (Catarino *et al.*, 2015; Kumar *et al.*, 2006; Yang *et al.*, 2013). As it is possible to observe in figure **12**, the increase of the samples concentration led to the consequent increase of their scavenging activity in the DPPH• and ABTS•+ and OH• assays.

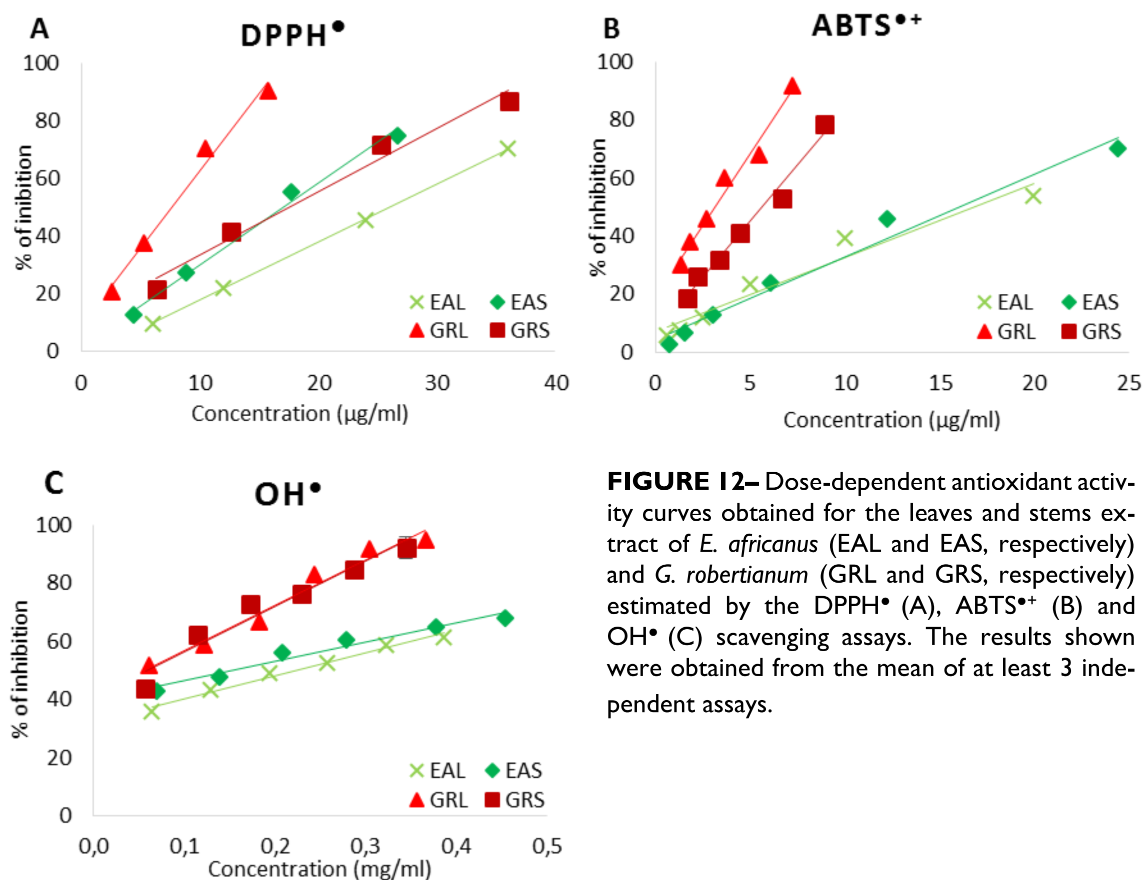


FIGURE 12– Dose-dependent antioxidant activity curves obtained for the leaves and stems extract of *E. africanus* (EAL and EAS, respectively) and *G. robertianum* (GRL and GRS, respectively) estimated by the DPPH• (A), ABTS•+ (B) and OH• (C) scavenging assays. The results shown were obtained from the mean of at least 3 independent assays.

Interestingly in all the experiments, GRL extract repeatedly demonstrated the highest antioxidant potential. In agreement to the foregoing, the IC_{50} values obtained in DPPH• and ABTS•+ scavenging assays for this extract (7.6±0.6 and 3.9±0.6 µg/mL, respectively) were statistically similar to those of the respective standard compounds (7.1±0.3 and 1.3±0.2 µg/mL, respectively) and much lower (45.1±2.4 µg/mL) than that of the mannitol (196.2±16.4 µg/mL) in OH• scavenging assay (Fig. **13**). Notably, a parity of the results obtained for GRL and GRS

was noted in $ABTS^{\bullet+}$ assay, in which the GRS extract also exhibited a good scavenging activity, though slightly lower than the corresponding leaves extract, and in $OH^{\bullet}$ assay where, together with GRL, it revealed great antioxidant potential with an IC_{50} three times lower (59.8±8.4 μg/mL) than that of mannitol. The only exception was in the $DPPH^{\bullet}$ assay in which GRS had a significantly lower activity (IC_{50} 17.3±0.3 μg/mL) compared to GRL.

Importantly the lower the IC_{50}, the less concentration is needed to inhibit 50% of the initial radical concentration and thus, low IC_{50} values indicate an elevated antioxidant potential for the samples.

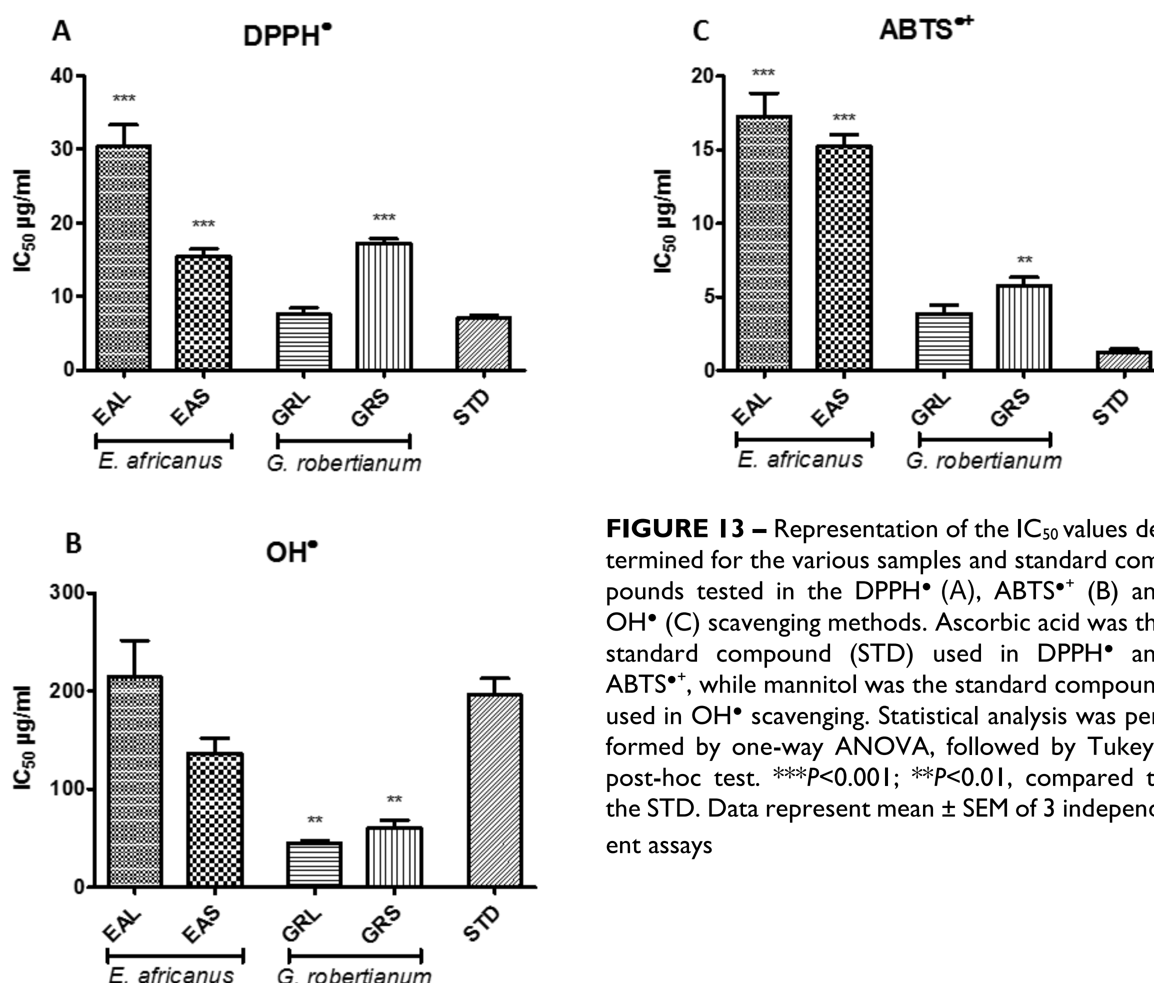


FIGURE 13 – Representation of the IC_{50} values determined for the various samples and standard compounds tested in the $DPPH^{\bullet}$ (A), $ABTS^{\bullet+}$ (B) and $OH^{\bullet}$ (C) scavenging methods. Ascorbic acid was the standard compound (STD) used in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, while mannitol was the standard compound used in $OH^{\bullet}$ scavenging. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***$P<0.001$; **$P<0.01$, compared to the STD. Data represent mean ± SEM of 3 independent assays

Interestingly, EAS extract had an identical IC_{50} value to that of GRS extract in $DPPH^{\bullet}$ assay, corresponding approximately to twice the antioxidant activity exhibited by ascorbic acid, which indicates that, though not as potent as GRL extract, their scavenging activity for this radical is still quite good. The worse result observed was by far the one obtained for EAL extract (IC_{50} of 30.4±1.7 μg/mL).

Paired results were also observed for EAL and EAS extracts in $ABTS^{\bullet+}$ and $OH^{\bullet}$ scavenging assays. However, if in the former assay both extracts exhibited significantly lower activities compared to the standard compound (IC_{50} 17.2±1.6, 15.3±0.8 µg/mL and 1.3±0.2 µg/mL, for EAL, EAS and ascorbic acid, respectively), in $OH^{\bullet}$, they have revealed IC_{50} values of 214.2±37.5 and 136.8±15.3 µg/mL, which were rather similar to that of mannitol (196.2±16.4 µg/mL), being noteworthy the tendency of a better scavenging capacity for the stems extract with respect to that of leaves.

From an overall perspective all the four samples exhibited good antioxidant activities as free radical scavengers, but *G. robertianum* extracts, and in particular the one from the leaves, demonstrated to be predominantly better than *E. africanus* extracts.

4.1.2 Ferric reducing antioxidant power and lipid peroxidation inhibitory capacities

Ferric reducing power and lipid peroxidation inhibitory capacity methods allow to measure the antioxidant potential of the plant extracts by determining their ability to reduce heavy metals and to inhibit the oxidative degradation of lipids, respectively (Ayala, Muñoz & Argüelles, 2014; Benzie & Strain, 1996).

As shown in figure **14**, in these two assays, the plant extracts also exerted a dose-dependent effects.

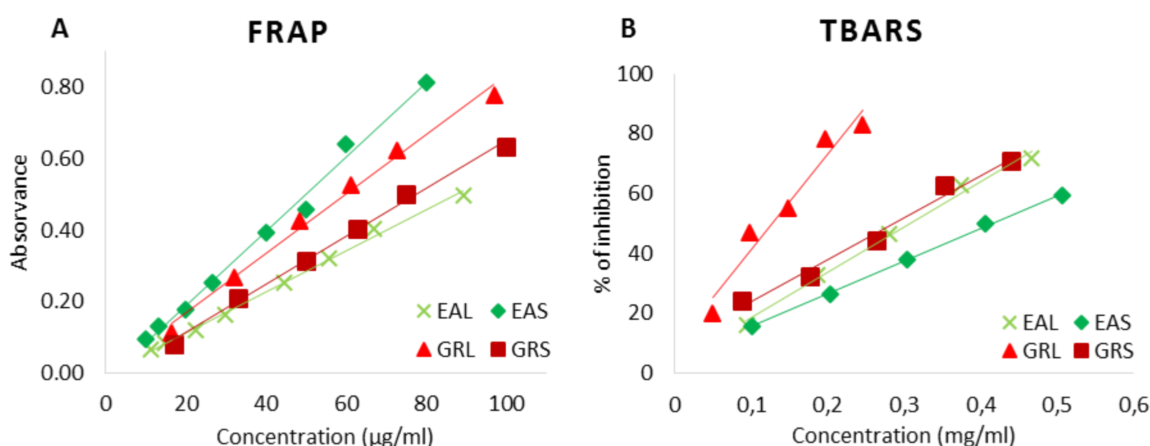


FIGURE 14 – Dose-dependent antioxidant activity curves obtained for leaves and stems extract of *E. africanus* (EAL and EAS, respectively) and *G. robertianum* (GRL and GRS, respectively), estimated by the FRAP (A) and TBARS (B) assays. The results shown were obtained from the mean of at least 3 independent assays.

In this case, a parity can be observed between the stems and leaves extracts of the different plants (see fig. **15A**), i.e., the IC_{50} from GRL extract was similar to that of EAS extract (63.3±5.4 and 61.5±1.3 µg/mL, respectively), while the IC_{50} from GRS was close to that of EAL extract (93.5±5.5 and 88.0±3.0 µg/mL, respectively). These values indicate approximately a 3

to 4-fold less general capacity for reducing Fe^{3+} to Fe^{2+} than that of the standard compound (IC_{50} 20.0±0.2 μg/mL).

In turn, GRL and GRS extracts were the most promising samples in TBARS assay, with IC_{50} values of 115.8±16.1 and 210.4±38.6 μg/mL against the 41.1±5.2 μg/mL obtained for trolox (fig. **15B**).

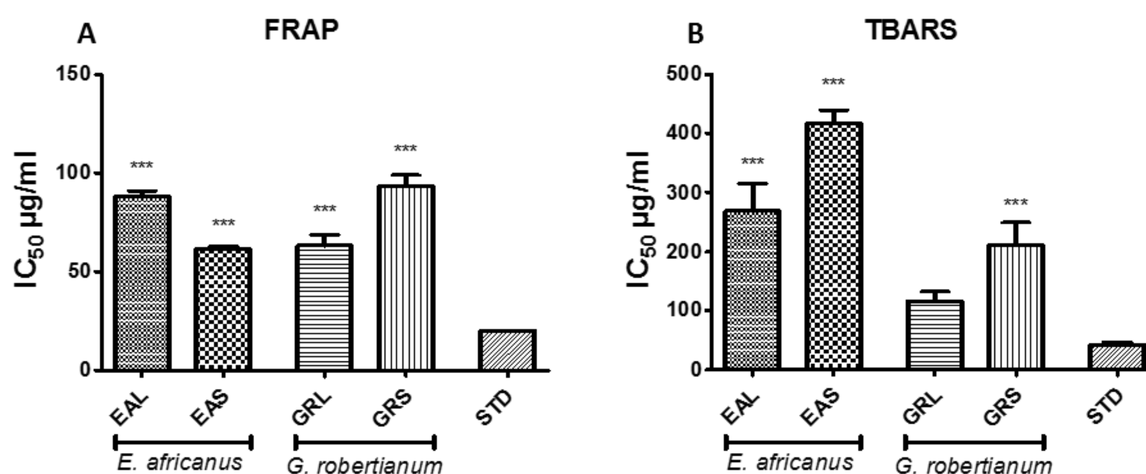


FIGURE 15 – Representation of the IC_{50} values determined for the various samples and standard compounds tested in the FRAP (A) and TBARS (B) assays. BHT was the standard compound (STD) used in FRAP, while trolox was the standard compound used in TBARS. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001, compared to the STD. Data represent mean ± SEM of 3 independent assays

Interestingly, in this assay the EAL extract has clearly demonstrated an higher IC_{50} value compared to the correspondent stems extract (268.6±45.9 against 416.1±237 μg/mL), thus indicating its better protective effect against lipid oxidative damage.

4.1.3 Oxygen radical absorbance capacity

In this assay, it was evaluated the extracts ability to block the oxidative degradation of fluorescein, and consequently loss of fluorescence. The antioxidant-dose response curves obtained by plotting the Net AUC, calculated from the kinetic curves, against the samples concentration is represented in figure **16**, and the respective ORAC values (expressed in μM TE) are listed in Table **3**. All the four samples exhibited good antioxidant activity but notably, EAS extract stands out with approximately twice the activity of the remaining samples, corresponding to 4.01±0.3 μM TE. In this case, GRL extract presented no significant differences from the two remaining samples, though through the figure **16** it is observable a slight tendency for its higher potential.

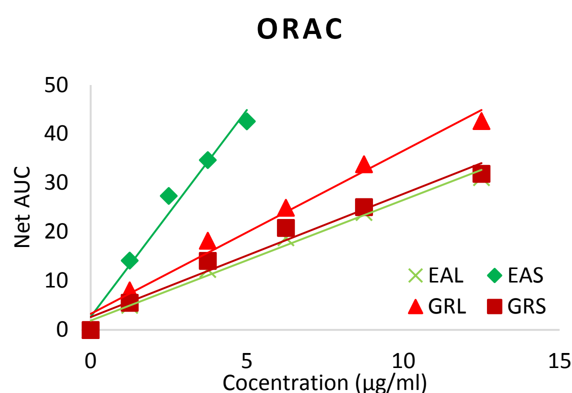


FIGURE 16 – Dose-dependent antioxidant curves obtained for the leaves and stems extracts of *E. africanus* and *G. robertianum* estimated through the ORAC assay. The results shown were obtained from the mean of at least 3 independent assays.

TABLE 3 – ORAC values obtained for the four samples tested

Sample	ORAC values (µM TE)
EAL	1.2±0.0***
EAS	4.01±0.3
GRL	1.8±0.1***
GRS	1.3±0.0***

Data expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. Each letter mean significant differences ***P<0.001, compared to the EAS extract.

4.1.4 NO• scavenging activity in chemical model

The screening of the scavenging ability of chemically-generated NO• is also an important method for establishment of the antioxidant abilities of samples. Still, as this radical is a key inflammatory mediator, the measurement of NO• scavenging capacity in chemical models is also used as a first general approach to evaluate the anti-inflammatory potential of the samples. Therefore, the four samples were tested in this assay not only to prove their antioxidant properties but also as a primary screening of their anti-inflammatory potential.

As can be observed in figure **17**, *G. robertianum* extracts were both undoubtedly extremely effective in the scavenging of this radical with IC_{50} values (20.0±0.9 and 24.2±8.0 µg/mL for GRL and GRS, respectively) of approximately 14 and 12 times lower, respectively, compared to that of the standard compound (285.7±15.4 µg/mL) (see fig. **17C**). These results indicate that these two samples are very potent NO• scavengers, suggesting that they might have significant anti-inflammatory activity.

On the other hand, both *E. africanus* extracts exhibited considerable lower capacity for the scavenging of NO•, with IC_{50} values of 526.4±22.5 and 538.6±16.4 µg/mL for EAL and EAS extracts, respectively.

Therefore, taking all this data together and since EAL extract globally revealed the less promising effects, it was excluded from further research, and the study proceeded with EAS and both *G. robertianum* extracts.

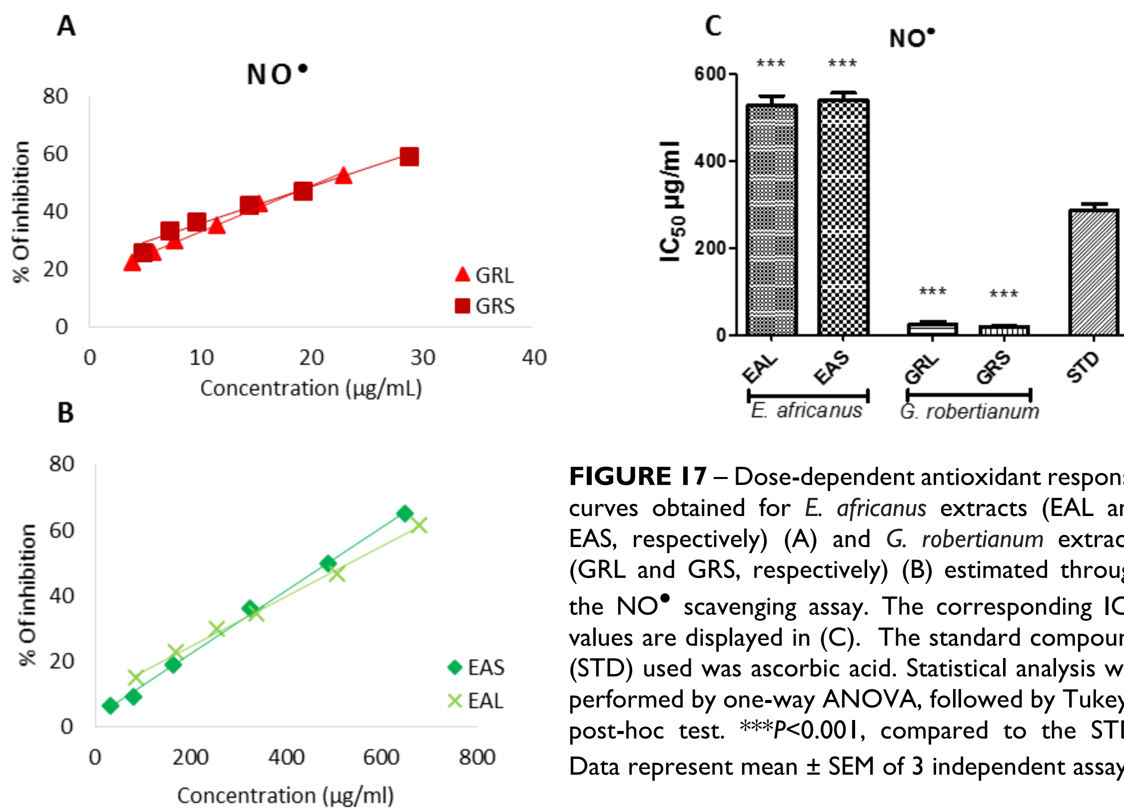


FIGURE 17 – Dose-dependent antioxidant response curves obtained for *E. africanus* extracts (EAL and EAS, respectively) (A) and *G. robertianum* extracts (GRL and GRS, respectively) (B) estimated through the NO• scavenging assay. The corresponding IC_{50} values are displayed in (C). The standard compound (STD) used was ascorbic acid. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***$P<0.001$, compared to the STD. Data represent mean ± SEM of 3 independent assays

4.2 *Evaluation of hepatocytes viability*

Safety assessment is an important aspect of toxicology for the development of novel pharmacological substances. For this reason, before proceeding to the anti-inflammatory analysis in the biological models, the putative hepatotoxicity of the samples were evaluated in a human hepatic cell line HepG-2 cells.

As shown in figure **18**, the *in vitro* effect of the extracts on HepG-2 viability was represented as percentage of the control (non-treated cells). In all samples tested, no significant cytotoxic effects were seen for any of the concentrations tested, indicating that these extracts are armless at the concentrations tested.

Nevertheless, it was noticed a slight tendency for decreasing the cell viability after treatment with GRL extract, despite not statistically significant (fig. **18B**).

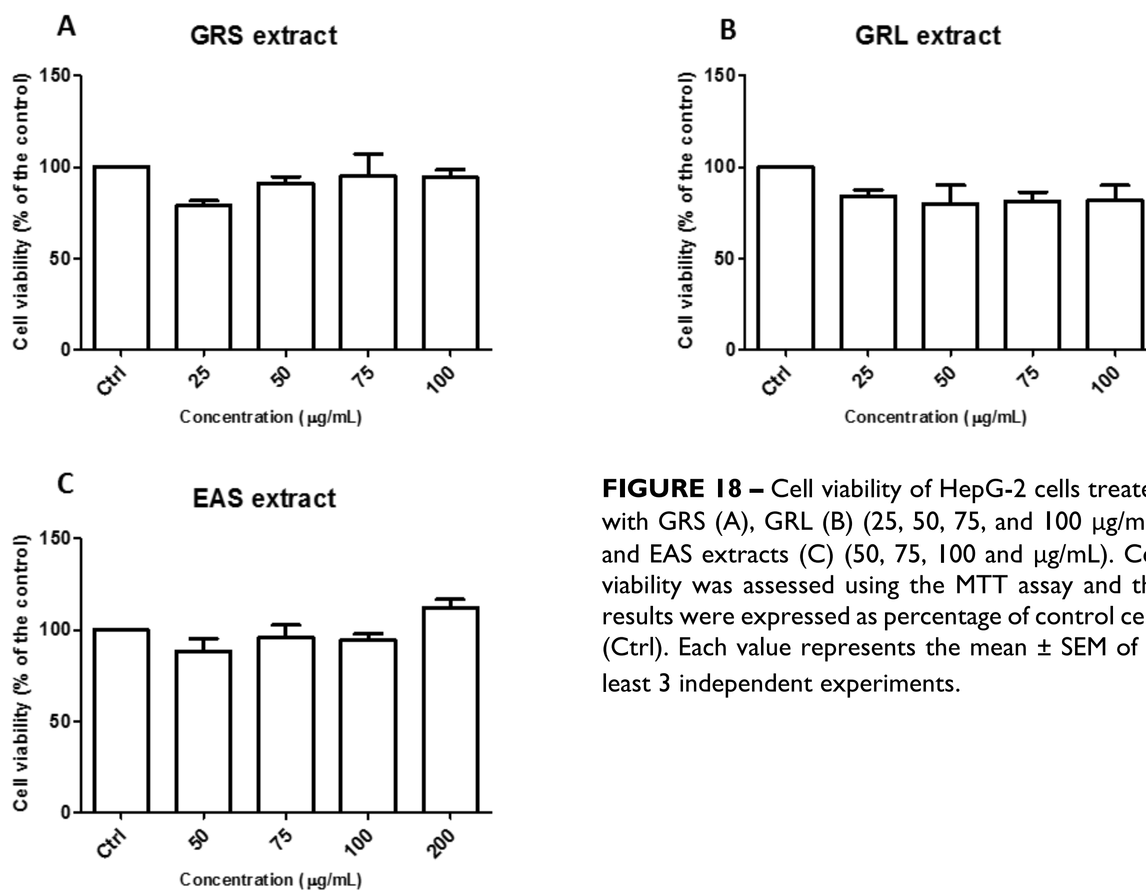


FIGURE 18 – Cell viability of HepG-2 cells treated with GRS (A), GRL (B) (25, 50, 75, and 100 µg/mL) and EAS extracts (C) (50, 75, 100 and µg/mL). Cell viability was assessed using the MTT assay and the results were expressed as percentage of control cells (Ctrl). Each value represents the mean ± SEM of at least 3 independent experiments.

4.3 Determination of anti-inflammatory activities

4.3.1 Effects on soybean 5-Lipoxygenase

5-LOX is an enzyme that catalyzes the oxidation of the arachidonic acid to produce leukotrienes which is another group of chemical mediators involved in inflammatory events and closely related to the pathological symptoms of asthma (Baylac & Racine, 2003; Cardoso *et al.*, 2014). Therefore this enzyme is considered an important pharmacological target for intervention in these events.

Looking at the kinetics of the reaction (fig. **19A**) it is possible to understand the effect of the extracts on the activity of 5-LOX. Both *G. robertianum* samples at concentrations of 75 µg/mL have shown a very similar inhibitory activity, wherein the slope of the kinetic curves were approximately 2.5 times lower than that of the control (with no inhibitors), corresponding to an inhibition of 64.4±2.9 and 63.5±1.4 % of the normal enzyme activity (fig. **19B**). Notably, these samples have shown to be as effective as the standard compound in the same concentration which has revealed 70.9±1.0 % inhibition of 5-LOX activity.

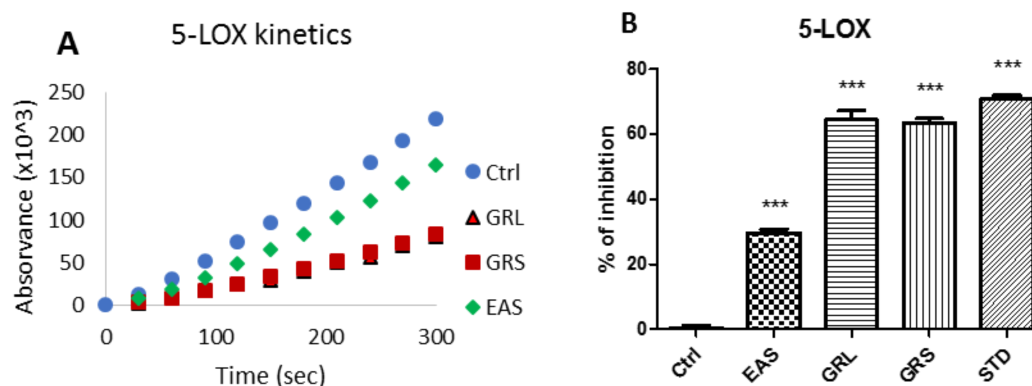


FIGURE 19 – Kinetic curves of the 5-LOX activity in absence or presence of 75 μg/mL of the samples studied (A) and their correspondent percentages of inhibition (B). The standard compound (STD) used was ascorbic acid. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***$P<0.001$, compared to the control (Ctrl). Data represent mean ± SEM of 3 independent assays.

The inhibitory activity exhibited by the EAS extract (also at 75 μg/mL) was less pronounced than those mentioned before. This extract was able to inhibit the 5-LOX activity in 29.7±1.1 %, which corresponds to approximately half of the effectiveness of the remaining samples. Yet, the inhibitory effect of this sample can still be considered relevant.

4.3.2 Effects on NO• production and cell viability on macrophages RAW 264.7

NO• is a signaling molecule resultant from the conversion of arginine into citrulline by iNOS and responsible for the transduction of a variety of signals closely related with the pathogenesis of inflammation (Sharma, Al-Omran & Parvathy, 2007). Therefore, inhibition of this radical species beneficiates the decrease of inflammatory conditions.

As it is possible to depict from the figure **20**, under normal conditions, macrophages RAW 264.7 produce low levels of nitrites (approximately 0.6±0.2 μM). Upon stimulation of these cells with LPS for 24h, the production of nitrites increased up to about 24.2±0.5 μM.

Based on the positive effects previously observed regarding the scavenging ability of chemically-generated NO•, it was expected that the pre-treatment of the cells with the three most promising samples would attenuate the levels of nitrites production by the LPS-stimulated RAW 264.7 macrophages. Still, this was not observed for the EAS extract, as it did not influenced the NO• release for any of the tested concentrations (fig. **20D**). However, pre-treatment of non-stimulated cells with 50 and 75 μg/mL of this extract significantly potentiated cell viability (fig. **20A**), indicating an increase of the cell metabolism. Despite not statistically significant, there is also a tendency for these same concentrations to increase the metabolism of LPS-stimulated cells when compared to the untreated ones.

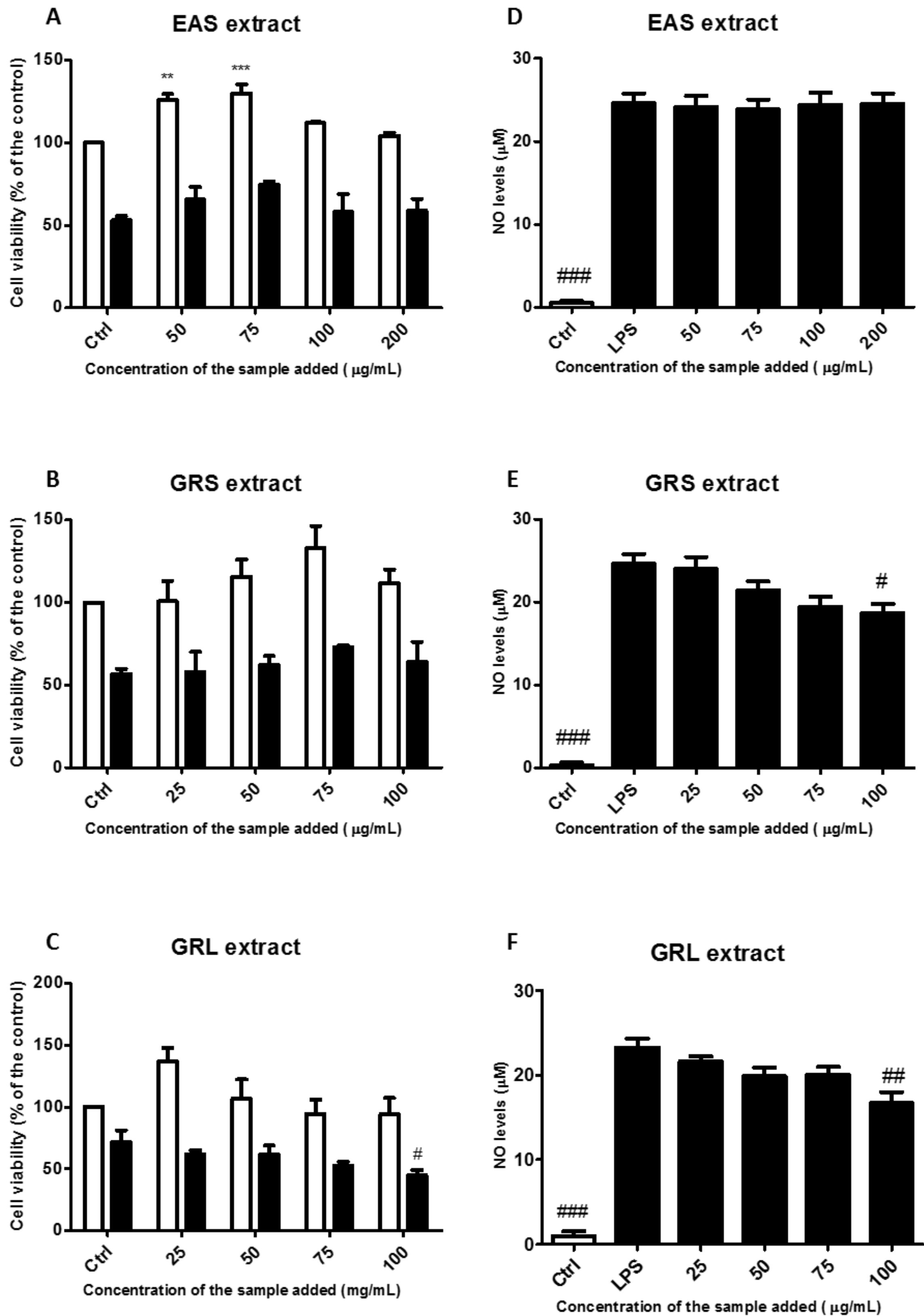


FIGURE 20 – Effects of the pre-treatment with EAS (50, 75, 100 and 200 µg/mL), GRS and GRL (25, 50, 75 and 100 µg/mL) on the cell viability (% of the control) (A, B and C, respectively) and NO• levels (µM) (D, E and F, respectively) on RAW 264.7 cells after 24h of incubation with (■) or without (□) LPS. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P*<0.01; ****P*<0.001, compared to the control without LPS; #*P*<0.05; ##*P*<0.01; ###*P*<0.001, compared to the control with LPS. Data represent mean ± SEM of 3 independent assays.

In turn, GRS and GRL extracts revealed significant inhibitory effects for the highest concentrations tested (100 μg/mL) (fig. **20E** and **F**). However, looking at figure **20C**, it is possible to conclude that the exposure of RAW 264.7 to GRL at this concentration also decreased cells viability, comparing to cells treated with LPS alone. Hence, the comparison of figures **20C** and **F** allowed to conclude that GRL does not exert an effective reduction of NO• release but instead, it caused cell death of macrophages.

Hence, the only promising results were obtained with GRS which was able to reduce the nitrites levels without interfering with cell viability (fig. **20B**). Therefore, this sample was subjected to Western blot analysis in order to deeply explore the molecular mechanisms behind its anti-inflammatory effect, specifically whether GRS extract could block the LPS-induced iNOS and COX-2 protein levels.

4.3.3 Effects on expression of iNOS and COX-2 enzymes

The effect of GRS on the expression of the enzymes iNOS and COX-2 are represented in figure **21**. As expected, in normal conditions, cells do not express neither iNOS nor COX-2. In turn, when the cells are stimulated with LPS the expression of this enzymes is triggered indicating that they entered in an inflammatory stage.

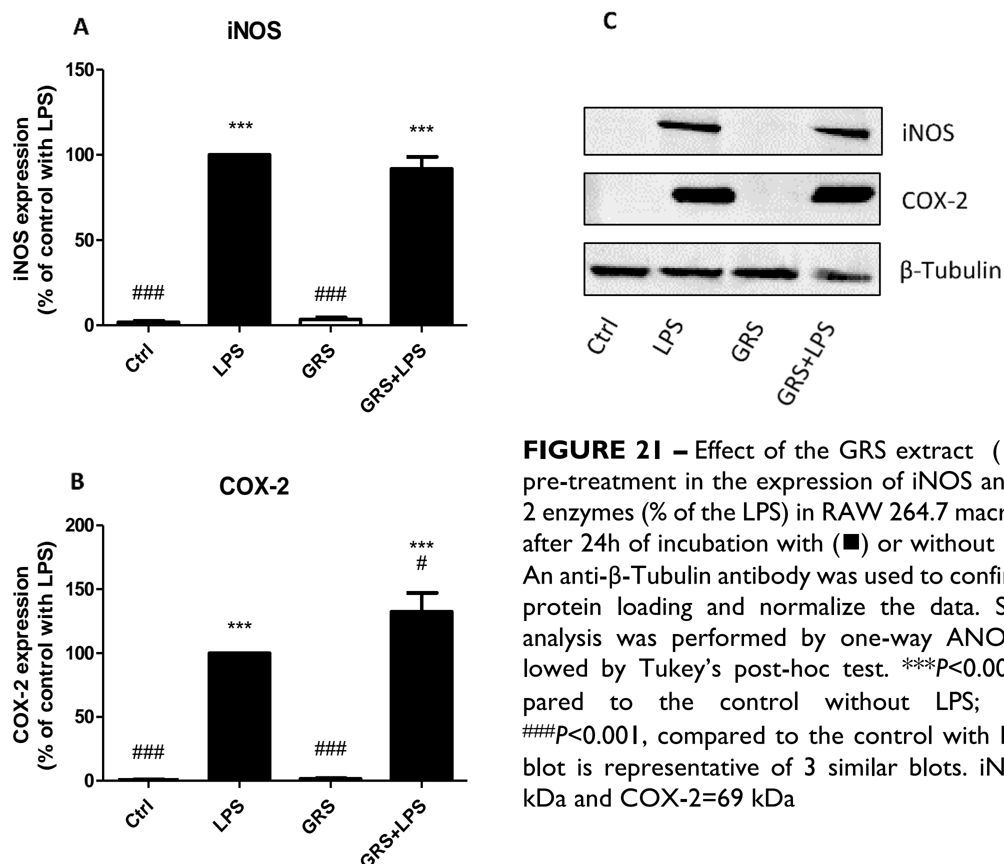


FIGURE 21 – Effect of the GRS extract (100μg/L) pre-treatment in the expression of iNOS and COX-2 enzymes (% of the LPS) in RAW 264.7 macrophages after 24h of incubation with (■) or without (□) LPS. An anti-β-Tubulin antibody was used to confirm equal protein loading and normalize the data. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. $^{***}P<0.001$, compared to the control without LPS; $^{\#}P<0.05$; $^{\#\#\#}P<0.001$, compared to the control with LPS. The blot is representative of 3 similar blots. iNOS=135 kDa and COX-2=69 kDa

The results depicted in figure **21** demonstrated that pre-treatment of RAW 264.7 with 100 µg/mL of GRS extract did not decrease the protein levels of the enzymes.

4.4 Identification and quantification of phenolic constituents from E. africanus and G. robertianum

The hydroethanolic EAS and EAL extracts represented 15 and 13% of the dried plant material, respectively while the correspondent extracts from *G. robertianum* accounted for 18 and 15% of dried stems and leaves of the plant, respectively.

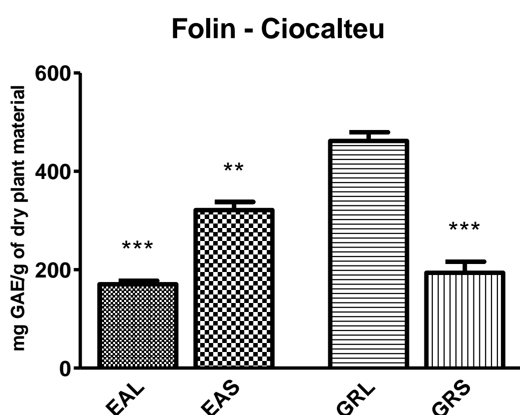


FIGURE 22 – Quantification of the total phenolic content on *E. africanus* leaves and stems (EAL and EAS, respectively) and *G. robertianum* leaves and stems extracts (GRL and GRS, respectively). Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001; **P<0.01, compared to GRL. Data represent mean ± SEM of 3 independent assays.

The results obtained from Folin – Ciocalteu method revealed that the total amount of phenolics recovered for *E. africanus* was higher in stems (321 mg GAE/g of dry material) than for leaves (171 mg GAE/g of dry material), while *G. robertianum* exhibited a reversed panorama with higher recovery of phenolic compounds in the leaves extract (462 mg GAE/g of dry material) with regard to that of stems (194 mg GAE/g of dry material) (fig. **22**). Looking from an overall perspective, these results indicate that, despite both plants are rich in phenolic compounds, *G. robertianum* as a whole plant clearly stands out when compared to *E. africanus*.

Overall, the HPLC–DAD–MS^n analysis allowed to conclude that, despite the variances in the peak intensities, both leaves and stems extracts of each plant demonstrated similar chromatographic profiles between each other, i.e., the composition of the leaves extracs were similar to that of the stems extracts within the same plant.

E. africanus extracts were particularly enriched in mono- and dicaffeoylquinic acids which accounted for approximately 90% and 74% of the total quantified phenolics for stems and leaves, respectively. On the other hand, *G. robertianum* extracts were more abundant in gallic and ellagic acid derivatives.

The chromatographic profiles for the two plants can be consulted in figures **23** and **24**, and in the tables **4** and **5**, respectively. A more detailed description of their phenolic composition will be further discussed in below.

4.4.1 Phenolic constituents in E. africanus extracts

Seven distinct caffeic acid derivatives were detected in the hydroethanolic extracts of stems and leaves of *E. africanus*, constituting the majority of the identified compounds (fig. **23** and table **4**). These compounds eluted at 9.7, 13.4, 20.9, 21.9, 22.9, 24.0 and 24.9 min showed characteristic UV spectra consistent to that described for caffeic and caffeoylquinic acid derivatives (Schütz *et al.*, 2004; Weisz, Kammerer & Carle, 2009). Of those, the major representative constituents of both extracts were eluted at 13.4 and 24.0 min, respectively.

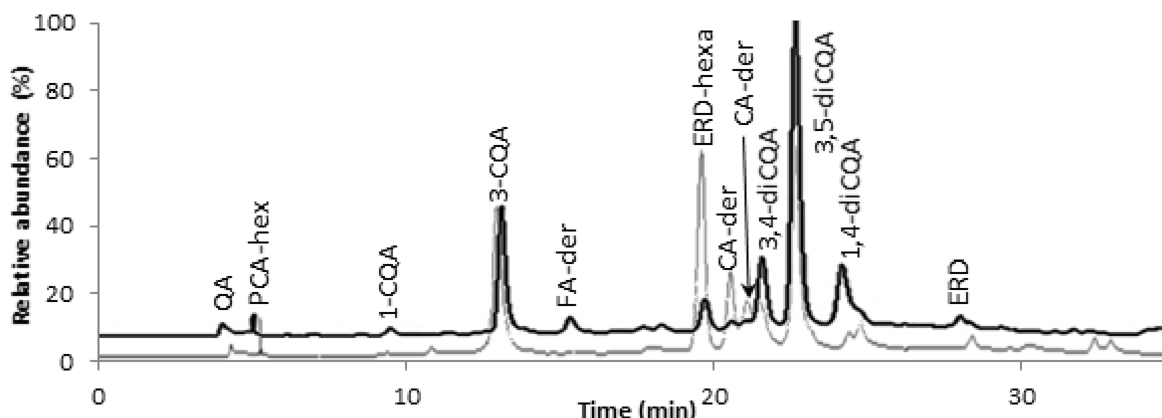


FIGURE 23 – Chromatographic profile of *E. africanus* hydroethanolic extracts at 280 nm. Chromatogram corresponding to stems extract is represented in bold lines, while the thin lines represent the chromatogram corresponding to the leaves extract. QA – quinic acid; PCA-hex – protocatechuic acid-hexoside; 1-CQA – 1-caffeoylquinic acid; FA-der – ferulic acid derivative; ERD-hexa – eriodictyol-hexuronide; CA-der – caffeic acid derivative; 3,4-diCQA – 3,4-dicaffeoylquinic acid, 3,5-diCQA – 3,5-dicaffeoylquinic acid; 4,5-diCQA – 4,5-dicaffeoylquinic acid; ERD – eriodictyol.

The compound eluting at 13.4 min was identified as a mono-caffeoylquinic acid and was assigned to chlorogenic acid i.e., 3-*O*-caffeoylquinic acid, since its retention time, MS data and corresponding MS^2 fragmentation pattern were in agreement with that of the standard compound. Notably, for both *E. africanus* extracts, 3-*O*-caffeoylquinic acid represented approximately 17% of the quantified phenolics and its total recovery was similar for stems and leaves (7.3 ± 0.1 and 6.9 ± 0.3 mg/g dried stems and leaves, respectively), thus suggesting that this phenolic compound is equally distributed in these two plant organs. In turn, the isomeric form of this compound, i.e. 1-caffeoylquinic acid (with retention time of 9.7 min), was only detected as a minor constituent in both extracts with slight prevalence in the stems.

The other prevalent caffeoylquinic acid derivative (eluted at 24.0 min) was highly recovered from the EAS extract (19.4 ± 0.2 mg/g dried plant material) than from the EAL extract (16.3 ± 0.1 mg/g dried plant material). The ESI-MS spectrum of this compound revealed a base peak at *m*/*z* 515, with a main MS^2 product ion at *m*/*z* 353 (−162 Da, loss of caffeoyl moiety), which in turn showed main fragments on MS^3 spectrum at *m*/*z* 191 (−162 Da, loss of other caffeoyl moiety), and at *m*/*z* 135. According to this features, this compound was assigned as

3,5-dicaffeoylquinic acid which together with two other dicaffeoylquinic isomers (MW 516 Da) that eluted at 22.9 and 24.9 min, accounted for about 71% and 56% of the total quantified phenolics in the hydroethanolic extracts of stems and leaves, respectively. The UV–vis spectrum of these two other compounds, as well as the fragmentation pattern of its molecular ion at *m*/*z* 515 and the corresponding MS^2 pattern (which showed a base peak ion at *m*/*z* 353), was similar to that of 3,5-dicaffeoylquinic acid. However, differences were evident on the HPLC retention times and MS^3 fragmentation patterns. Both MS^3 spectra of the ion at *m*/*z* 353 eluting at 22.9 and 24.9 min showed a base peak ion at *m*/*z* 173, which indicates the presence of a caffeoyl moiety bonded to quinic acid at the position 4 (Clifford *et al.*, 2003). It is also known that, among the three dicaffeoylquinic acid isomers that exist in nature with this feature (3,4-diCQA, 1,4-diCQA and 4,5-diCQA), both 1,4-diCQA and 4,5-diCQA elute after 3,5-diCQA under HPLC reversed-phase conditions (Clifford *et al.*, 2003; Schütz *et al.*, 2004; Weisz, Kammerer & Carle, 2009). Moreover, 1,4-diCQA is the only one showing the product ions at *m*/*z* 299, *m*/*z* 203 and *m*/*z* 255 in the MS^2 spectrum of the ion at *m*/*z* 353 (Clifford, Knight & Kuhnert, 2005). By these reasons, the compound detected at 24.9 min could be assigned to 1,4-diCQA. On the other hand, the compound eluting at 22.9 min was identified as 3,4-diCQA, since it eluted before 3,5-diCQA, such as described in the literature for HPLC reversed-phase conditions (Schütz *et al.*, 2004). The concentrations of the above identified diCQA isomers (3,4-diCQA and 1,4-diCQA) were similar within the same extract, yet, their overall recovery was higher in the stems extract (5.6 ± 0.2 and 5.4 ± 0.1 mg/g dried stems, respectively) than that in the leaves (4.0 ± 0.2 and 3.7 ± 0.1 mg/g dried leaves, respectively).

Notably, *E. africanus* extracts also contained two caffeoyl-hexuronide derivatives (with retention times of 20.9 and 21.9 min), which were substantially abundant in the EAL extract (1.6 ± 0.1 and 1.1 ± 0.0 mg/g dried plant material, respectively), while only vestigial amounts of these compounds were found in the EAS extract. Both compounds had a molecular ion of 518 Da and possessed equivalent UV–vis and fragmentation patterns on MS^n analysis. In particular, the base peak ion in the MS^2 spectrum was observed at *m*/*z* 355 (−162 Da, i.e., equivalent to the loss of a caffeoyl moiety or of a hexose). Moreover, the MS^3 spectrum of the latter ion corroborated the hypothesis of a caffeoyl-hexuronide derivative, as characteristic fragment ions of caffeic acid (at *m*/*z* 179 and *m*/*z* 135) and of an hexuronide acid (at *m*/*z* 175 and *m*/*z* 113) were registered (Bastos, Saldanha & Catharino, 2007; Gu, Zhong & Chen, 1999).

TABLE 4 – Identification of LC-DAD-ESI/MS^n data, and quantification of the most relevant fractions from the extracts of *E. africanus*.

RT (min)	λ_{max}	Compound (MW)	Main fragment ESI$^-$ MS^n	mg/g dried material[a]		Compound
				Stems	Leaves	
4.2	269	192	MS^2[191]: 173	-	-	Quinic acid
5.3	279	316	MS^2[315]: 153; MS^3[153]: 109	0.4±0.0	-	Protocatechuic-acid-4-gluco-side
9.7	324	354	MS^2[353]: 191; MS^3[191]: 173, 109	-	-	1-Caffeoylquinic acid
13.4	219, 239, 325	354	MS^2[353]: 191, 179	7.3.±0.1	6.9±0.3	3-Caffeoylquinic acid
15.6	324	560	MS^2[559]: 193; MS^3[193]: 134	1.5±0.0	-	Ferulic acid derivative
19.9	283	464	MS^2[463]: 287, 151; MS^3[287]: 151; MS^4[151]: 107	1.6±0.1	7.8±0.1	Eriodictiol-*O*-hexuronide
20.9	318	518	MS^2[517]: 355, 311, 179; MS^3[355]: 311, 179, 175, 113, 135; MS^4[311]:175, 113, 135	-	1.6±0.1	Caffeoyl-hexuronide deriva-tive
21.9	319	518	MS^2[517]: 355, 311, 473, 293, 179 ; MS^3[355]: 311, 179, 175, 113, 135; MS^4[311]:175, 113, 135, 251, 293	-	1.1±0.0	Caffeoyl-hexuronide deriva-tive
22.9	217, 241, 325	516	MS^2[515]: 353; MS^3[353]: 173, 179, 191, 135; MS^4[173]: 111, 155	5.6±0.2	4.0±0.2	3,4- Dicaffeoylquinic acid
24.0	218, 326	516	MS^2[515]: 353; MS^3[353]: 191, 135; MS^4[191]: 173, 127	19.4±0.2	16.3±0.1	3,5- Dicaffeoylquinic acid
		610	MS^2[609]: 301; MS^3[301]: 286, 213	-	-	Hesperetin
24.9	218, 242, 327	516	MS^2[515]: 353; 203; 299; 255; 173; 179 MS^3[353]: 173, 179	5.4±0.1	3.7±0.1	1,4- Dicaffeoylquinic acid
28.4	228, 288	288	MS^2[287]: 151	0.2±0.0	0.2±0.0	Eriodictyol

[a] Data represent the mean values ± S.E.M.

Hence, albeit the overall structural information of these two isomers could not be achieved, the gathered information allow to conclude that these are caffeoyl-hexuronide with an additional caffeic acid and/or hexose moiety.

Besides the caffeic acid derivatives, other phenolic compounds were detected in low amounts in the hydroethanolic extracts of *E. africanus*. These included protocatechuic acid-hexoside ([M−H]− at *m/z* 315, eluted at 5.3 min) and a ferulic acid derivative ([M−H]− at *m/z* 560, eluted at 15.6 min) that overall accounted for 1.9 mg/g of dried stem and only appeared as vestigial components in leaves.

Flavonoids in *E. africanus* only comprised flavanones. Of these, hesperetin (co-eluted at 24.0 min) and eriodictyol (eluted at 28.4 min) were detected as minor components in the extracts of the two plant organs. The most representative flavanone eluted at 19.9 min and was assigned to eriodictyol-*O*-hexuronide since its MS data and fragmentation profile were consistent to those reported in the literature (Manach *et al.*, 2004; Pereira *et al.*, 2013). Interestingly, its abundance was clearly more pronounced in the EAL extract with regard to that of EAS extract (7.8 ± 0.1 and 1.6 ± 0.1 mg/g dried material, respectively).

4.4.2 Phenolic constituents of G. robertianum extracts

Nine compounds have been identified in *G. robertianum* extracts, wherein five were assigned to ellagic acid and ellagitannin derivatives. These compounds eluted at 10.2 (co-eluted with chlorogenic acid), 16.5, 17.4, 20.4 and 33.9 min, and together constitute the majority of the identified compounds in these samples (fig. **24**, table **5**)

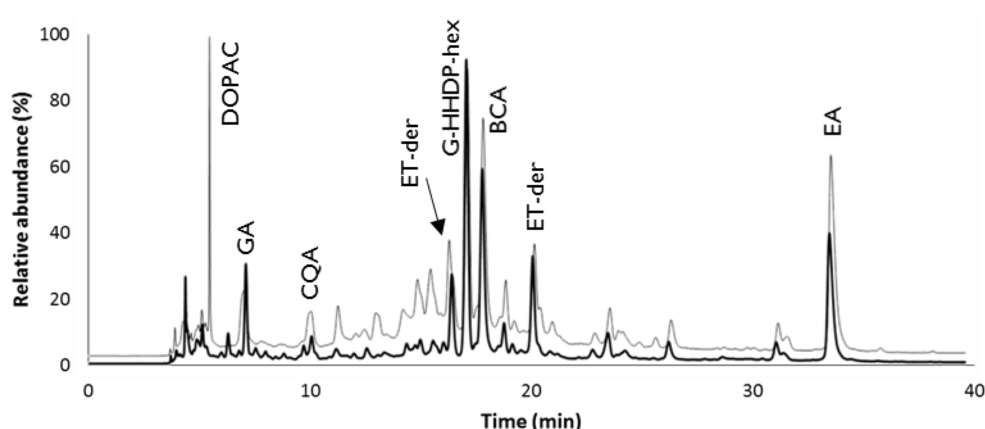


FIGURE 24 – Chromatographic profile of *G. robertianum* hydroethanolic extracts at 280 nm. Chromatogram corresponding to stems extract is represented in bold lines, while the thin lines represent the chromatogram corresponding to the leaves extract. DOPAC – 3,4-dihydroxyphenylacetic acid; GA – gallic acid; CQA – caffeoylquinic acid; ET-der – ellagitannin derivative; G-HHDP-hex – Galloyl-HHDP-hexoside; BCA – brevifolin carboxylic acid; EA – ellagic acid.

The compound eluted at 33.9 min was easily identified as ellagic acid since it eluted with high percentages of acetonitrile and its UV spectra together with the MS fragmentation patterns were in agreement to literature data (Fischer, Carle & Kammerer, 2011; Lee, Johnson & Talcott, 2005; Meyers, Swiecki & Mitchell, 2006). Galloyl-HHDP-hexoside has also been detected in *G. robertianum* extracts. In fact, this compound was detected at two different eluting times, the first co-eluted at 10.2 min and the second at 17.4 min, which indicates that they are two isomeric forms of the same compound.

The fragmentation of these two compounds (base peak at *m/z* 633) have released the fragment at *m/z* 301 that typically indicates the presence of ellagic acid. Moreover, the fragmentation pattern as well as the UV spectra of these compounds are consistent to previous reports (Fischer, Carle & Kammerer, 2011; Mena *et al.*, 2012).

The two remaining ellagitannin derivatives were eluted at 16.5 and 20.4 min. Although the exact identification of these compounds was not achieved, the MS^2 fragmentation revealed the presence of the fragment at *m/z* 301 indicating the presence of ellagic acid. Besides, looking at the UV spectra of these compounds it is possible to note a similarity to that of the ellagic acid (Fracassetti *et al.*, 2013; Mullen *et al.*, 2003).

TABLE 5 – Identification of LC-DAD-ESI/MS^n data of the most relevant fractions from the extracts of *G. robertianum*.

RT (min)	λ_{max}	Compound (MW)	Main fragment $ESI^- MS^n$	Compound
5.5	285	168	MS^2[167]: 123	3,4-Dihydroxyphenylacetic acid
7.0	272	170	MS^2[169]: 125	Gallic acid
10.2	220, 325	354	MS^2[353]: 191, 179, 135	Caffeoylquinic acid
		634	MS^2[633]: 301, 463	Galloyl-HHDP- hexoside
16.5	274	485	MS^2[484]: 462, 169 MS^3[462]: 440, 169, 301, 364, 247	Ellagitanin derivative
17.4	267	634	MS^2[633]: 301, 463, 419	Galloyl-HHDP- hexoside
18.1	277, 355	292	MS^2[291]: 247	Brevifolin carboxilic acid
20.4	279	463	MS^2[462]: 453, 415, 169, 301	Ellagitannin derivative
33.9	279	302	MS^2[301]:257, 133, 284, 185	Ellagic acid

Notably, the compounds eluting at 7.0 and 18.1 min have also shown to be expressive in the *G. robertianum* extracts. The former revealed a UV/vis maxima at 172 nm and its full MS spectrum showed a base peak at *m/z* 169. Moreover, the product ion at *m/z* 125 (loss of carboxylic acid moiety) was obtained in the tandem fragmentation. Comparing this results with the data in the literature it is possible to clearly identify this compound as gallic acid (Lee, Johnson & Talcott, 2005). On the other hand, the fragmentation of the compound eluted at

18.1 min (base peak at *m*/*z* 291) originated a fragment ion at *m*/*z* 247 (again loss of carboxylic acid moiety) which is characteristic to that of brevifolin carboxylic acid (Antonia Alvarez-Fernadez *et al.*, 2015). Moreover the UV/vis maxima at 277 and 355 nm is also in agreement to what has been described for this compound (He & Xia, 2007).

Interestingly, caffeoylquinic acid was also present in the *G. robertianum* extracts ($[M-H]^-$ at *m*/*z* 353, eluted at 10.2 min). However this compound, together with the 3,4-dihydroxyphenylacetic acid ($[M-H]^-$ at *m*/*z* 168, eluted at 5.5 min) were only found in minor amounts.

DISCUSSION

5 DISCUSSION

Because of the health benefits that have been attributed to *E. africanus* and *G. robertianum*, these two plants have been traditionally used in the folk medicine for the treatment of several ailments. In this study, the cytotoxicity, antioxidant and anti-inflammatory properties of hydroethanolic extracts from these two plants were investigated, along with their phenolic composition, in order to disclose its potential for therapeutic and/or nutraceutical purposes.

The antioxidant abilities of leaves and stems extracts from both plant species were evaluated for their scavenging and reducing capacities, as well as the protective effects towards oxidative degradation of lipids or fluorescein.

In a general perspective, GRL extract was clearly the most promising sample regarding to the antioxidant abilities, showing low IC_{50} values in DPPH•, ABTS•+ and particularly in OH• scavenging assays (7.6±0.6, 3.9±0.6 and 45.1±2.4 µg/mL, respectively). In fact, in the latter assay its effect was even better than that of the standard compound (196.2±16.4). The GRS extract also demonstrated significantly good radical scavenging effects in these three assays, followed by the EAS extract. Opposing to GRL extract, the EAL extract was undoubtedly the one with the worst scavenging abilities, registering the highest IC_{50} values for all the three assays (30.4±1.7, 17.2±1.6 and 214.2±37.5 µg/mL for DPPH•, ABTS•+and OH•, respectively). Despite this, it is of noteworthy that the DPPH• scavenging activity herein estimated for the EAL extract was better than that previously reported by Njenga & Viljoen (2006) for the acetone extracts of *E. africanus* (IC_{50} values ranging from 38 to 50 µg/mL).

According to the HPLC–DAD–MS^n analysis, *E. africanus* extracts were enriched in caffeoylquinic acids (which accounted for approximately 90% and 74% of the quantified compounds in stems and leaves, respectively), while ellagitannins were the major compounds identified in *G. robertianum* extracts. It is worth noting that either chlorogenic acids or ellagitannins have proven strong antiradical activities (Hagerman *et al.*, 1998; Park, 2013) and hence, it is probable that these compounds are important contributing players for the overall activity evidenced in the present study. The herein gathered results also corroborate the relevance of phenolic compounds in the extracts' antiradical activities since the most promising sample, i.e., the GRL extract, was the most enriched in phenolic compounds (462 mg GAE/g of dry material), while the least active sample, i.e., EAL extract, was the poorest one (171 mg GAE/g of dry material).

Notwithstanding, this straight correlation between the phenolic content and antioxidant activity was not so evidenced in TBARS and ORAC assays for the two plants. E.g. in the first method, EAL extracts revealed better activity than that of the EAS extract, despite its poverty

in phenolic compounds with respect to the latter extract (268.6±45.9 against 416.1±237 μg/mL). Also, the EAS extract, which is less enriched in phenolic compounds than GRL, was undoubtedly the sample that more effectively protected fluorescein from the oxidative damage, revealing an ORAC value that represents approximately twice of the antioxidant potential of the remaining samples. These results suggest that other compounds that have not been considered (e.g. presence of non-phenolic compounds), could also contribute for the antioxidant activities of these extracts.

Another possible justification is that the different phenolic compounds present in each extract could be differently contributing for their antioxidant activities, since significant phenolic differences have been encountered. According to the HPLC-MS^n analysis, nine compounds were identified in *G. robertianum*, from which the major six corresponded to ellagitannins and respective basic units (gallic, ellagic and brevifolin carboxylic acids). The two remaining minor compounds were identified as caffeoylquinic acid and 3,4-dihydroxyphenylacetic acid. No flavonoids were detected in the extracts from this plant. On the other hand, twelve peaks have been identified in the *E. africanus* extracts, and seven of them matched with caffeoylquinic acid derivatives. Other phenolic acids, namely protocatechuic and ferulic acids were also present in minor quantities, and the only flavonoids identified were hesperetin and eriodictyol derivatives, both belonging to the flavanones group. The presence of flavonoids were more expressive in the EAL extract than in the stems, mainly as eriodictiol-*O*-hexuronide. Considering that eriodictyol has been reported previously as an effective inhibitor of TBARS (Areias *et al.*, 2001), it is possible that the EAL extract enrichment in a derivative of this flavanone with respect to the EAS extract can contribute for its stronger effects on TBARS assay.

Likewise, correlation between high caffeoylquinic acids content and good ORAC values have already been reported previously (Kayano *et al.*, 2002; Mullen *et al.*, 2011). In fact, strong ORAC results have also been described for the chlorogenic acids rather than for ellagic acid and ellagitannins, such as galloyl-HDDP or brevifolin (Ishimoto *et al.*, 2012). Since the EAS extract is particularly enriched with this type of compounds, this could be a possible explanation for the results obtained in ORAC assay. However, EAL extract that is also abundant in chlorogenic acids, did not revealed as good results as the stems extract, meaning that these compounds are not the only explanation for the effects on ORAC assay. In this case, it is probable that other non-phenolic compounds not identified in the EAS extract may also account for this result.

Among all antioxidant assays, FRAP was the one for which all the samples revealed the weakest results. Both GRL and EAS extracts simultaneously demonstrated superior ferrous

ion reducing capacity compared to the remaining samples. Still these results fall a little short when compared to BHT, indicating that reducing heavy metals might not be the primary pathway for their antioxidant properties.

NO• is one of the many chemical mediators produced by the cells of the immune system upon an inflammatory state, thus, inhibition of this radical counteracts the inflammatory process (Cardoso *et al.*, 2014). Therefore, testing samples ability to scavenge chemically-generated NO• is very useful approach not only for evaluating their antioxidant properties, but also as a primary screening of their potential anti-inflammatory capacities. The results have shown that both *G. robertianum* extracts were potent scavengers of NO•, with IC_{50} values 14 and 12 times lower (20.0±0.9 and 24.2±8.0 µg/mL for GRL and GRS, respectively) than that of the standard compound (285.7±15.4 µg/mL). On the other hand, the results obtained for *E. africanus* extracts were not so prominent since both extracts revealed approximately twice the IC_{50} values of ascorbic acid (526.4±22.5 and 538.6±16.4 µg/mL for EAL and EAS extracts, respectively). According to the literature, the scavenging effects of ellagic acid over NO• are stronger than those of chlorogenic acid (Rodrigues, Toledo Benassi & Bragagnolo, 2014; Srivastava, Jagan Mohan Rao & Shivanandappa, 2007) and hence this fact can partly explain the results herein described.

As the overall perspective, antioxidant assays pointed EAL extract as the least promising sample and because of that, it was then excluded from the subsequent assays.

Due to the functional features of the liver and their role in the metabolic elimination of xenobiotics, the evaluation of potential hepatotoxicity represents a critical step for attesting the safety of the samples in study (Gómez-Lechón *et al.*, 2010). In this context, the cell viability of human hepatic cell line HepG-2 treated with EAL, GRL and GRS extracts was evaluated, and the results revealed that no significant cytotoxic effects were exhibited for any of the concentrations tested (25 – 100 µg/mL for *G. robertianum* extracts and 50 – 200 µg/mL for EAS extract), thus attesting the safety profile of the extracts studied.

Since scientific data regarding the potential anti-inflammatory activities of *E. africanus* and *G. robertianum* are scarce, this work also investigated the potential benefic effects of these two plant extracts in inflammatory conditions. In this context, it was assessed whether these three extracts could interfere with the activity of isolated 5-LOX. Very promising inhibitory effects were obtained for *G. robertianum* extracts, which were able to inhibit approximately 65% of this enzyme activity at 75 µg/mL, while for the same concentration, the EAS extract only retarded the enzyme activity by about 30%.

Notably, it has been previously demonstrated that chlorogenic acids (at 20 μM) have no effects over 5-LOX. However, compounds like protocatechuic acid (at 20 μM), ferulic acid (at 50 μM) and eriodictyol (at 40 μM) were shown to be potent inhibitors of this enzyme (Lee et al., 2010; Maqsood & Benjakul, 2010; Ribeiro et al., 2014). Therefore, despite these three compounds are only present in minor concentrations, their combined activities could contribute for the slight inhibitory activity of EAS extracts over 5-LOX. Still, this hypothesis needs to be confirmed.

On the other hand, the effects of isolated ellagic and gallic acids on lipoxygenase are still poorly described. Several gallic acid derivatives have been reported as strong inhibitors of this enzyme (Richard-Forget *et al.*, 1995). Moreover, various extracts from different pomegranate flowers, which are particularly enriched in gallic and ellagic acid compounds, have also revealed promising effects on LOX activity (Bekir *et al.*, 2013). Therefore, it is reasonable to assume that these compounds might be responsible for the *G. robertianum* extracts effects over 5-LOX, although with this limited information it is not possible to clearly confirm this hypothesis. Thus, it would be necessary to test gallic and ellagic acids separately or alternatively, different extract fractions that correspond to the various compounds identified in *G. robertianum* extracts, in order to understand the contribution of the individual compounds and/or possible synergies on the inhibitory effects of these extracts against 5-LOX activity.

It is worth noting that this is the first time that *G. robertianum* extracts are described as inhibitors of 5-LOX activity. In turn, *E. africanus* has already been described as moderate inhibitor of this enzyme, but only essential oils were tested in that study (Njenga & Viljoen, 2006).

Taking this results into account, it is conceivable to suggest that *G. robertianum* extracts could be thought as potential pharmacological tools for the treatment of inflammatory disorders in which lipoxygenase plays a crucial role, including asthma, rheumatoid arthritis or inflammatory bowel disease.

Both chlorogenic and ellagic acids, as well as some of their derivatives, have been previously reported for their promising effects as anti-inflammatory agents, namely through inhibition of iNOS and $NO^{\bullet}$ release, COX-2 and even NF-κB (Umesalma & Sudhandiran, 2010; Zhang *et al.*, 2010). Moreover, given the results obtained in the chemical scavenging of $NO^{\bullet}$, particularly for the *G. robertianum* extracts, it was expected that these samples would demonstrate anti-inflammatory potential in biological systems, namely in RAW 264.7 cells. However, EAS extract showed no evident inhibitory effect on nitrite production by macrophages up to concentrations of 50 – 200 μg/mL. Despite this, it is interesting to note that macrophages

treatment with EAS extract potentiated the cell metabolism of non-stimulated cells at the concentrations of 50 and 75 μg/mL. This could mean an increase on the mitochondrial activity or increased cell proliferation, which would be interesting to exploit for tissue regeneration purposes. Either way, further studies would be necessary to exploit this possibility.

On the other hand, *G. robertianum* extracts showed a relevant inhibitory effect for the highest concentration (100 μg/mL) on the nitrites levels in LPS-stimulated macrophages. However, for the GRL extract, this treatment also induced a decrease in cell viability of about 30% and hence the observed decrement of nitrite levels are probably due to cell death instead of an anti-inflammatory effect. Still note that as no toxic effect was registered for the treatment of non-stimulated macrophages with GRL, the results suggest that this extract for itself at 100 μg/mL does not induce toxicity in the cells, but instead it might potentiate the toxicity of LPS.

The GRS extract was the only sample that reduced $NO^{\bullet}$ levels in RAW 264.7 without decreasing cells viability and therefore, in an attempt to disclose the molecular mechanisms behind its anti-inflammatory activity, the expression of iNOS and COX-2 enzymes were further analyzed by Western blot.

Previous studies have reported that ellagic acid was able to reduce the expression of both COX-2 and iNOS and even NF-κB in different *in vitro* and *in vivo* models (Larrosa *et al.*, 2010; Mansouri *et al.*, 2015; Umesalma & Sudhandiran, 2010). Therefore, it was expected that the GRS extract would exhibit similar results on RAW 264.7 cells. However, data obtained in this study showed that macrophages treatment with GRS extract at 100 μg/mL did not change the levels of iNOS. A possible justification is that the interactions between the compounds in the extract might be neutralizing the effects of ellagic acid, or alternatively the concentration that was added to the medium was insufficient to exert the desirable effects. It is worth noting that despite not influencing iNOS levels, the GRS extract was still able to decrease $NO^{\bullet}$ levels in LPS-activated macrophages. This indicates that, at 100 μg/mL, this sample possesses anti-inflammatory potential through the scavenging of free radicals.

All the gathered data suggest that, given the antioxidant effects demonstrated by both plants, they could be used as nutraceutical ingredients to enrich the basic nutritional food dietary with additional nutrients that would provide extra benefits to protect from the negative effects of the oxidative stress that humans are frequently subject. However, it is important to point out that chemical models herein used are far to accurately reproduce the effects that these samples might have in a biological environment. Therefore, the results presented in this work may only be considered as a primary screening for the samples antioxidant potential. To further corroborate this data, it would be highly desirable to perform additional biological

studies such as testing the samples in *in vitro* systems and even in *in vivo* animal models that clearly provide results closer to the reality.

Likewise, the effects on 5-LOX activity indicate that there is a strong potential for the *G. robertianum* extracts to be used as anti-inflammatory agents targeting this enzyme. Still, once again, further studies in *in vitro*/*in vivo* models are necessary to validate the herein presented results.

Regarding to the effects on the $NO^{\bullet}$ production, only *G. robertianum* extracts exhibited significant potential to inhibit the levels of this radical, but since GRL extract effects were correlated to cell death, only the results obtained for the GRS extract were considered relevant. Notwithstanding, after confirming that this extract did not affect the levels of iNOS enzyme, it was concluded that its capacity to reduce the nitrite levels was resultant from its ability to scavenge this radical, which is once again an antioxidant skill. Furthermore, since all the extracts studied have shown potent antioxidant abilities, evaluation of the intracellular production of ROS would be another possible approach to explore the beneficial effects that are attributed to these plants.

CONCLUSIONS AND FUTURE PERSPECTIVES

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The main goal of this study was to evaluate the antioxidant, anti-inflammatory and safety profile of *E. africanus* and *G. robertianum* hydroethanolic extracts, as well as to characterize their phenolic constituents in order to understand if there is potential for exploitation of these two plants as possible therapeutic and/or nutraceutical agents.

The antioxidant evaluation of the leaves and stems of *Eriocephalus africanus* (EAL and EAS, repectively) and *Geranium robertianum* (GRL and GRS, respectively) was carried out through DPPH•, ABTS•+, OH•, FRAP, TBARS, NO• and ORAC assays. It was observed that GRL extract exhibited the best scavenging abilities revealing IC_{50} values of 7.6±0.6, 3.9±0.6, 45.1±2.4, 63.3±5.4 and 115.8±16.1 µg/mL for DPPH•, ABTS•+, OH•, FRAP and TBARS assays respectively. The exception was in the ORAC assay where the EAS extract revealed the strongest antioxidant capacity (4.01±0.3 µM TE). EAL extract was shown to be the less promising sample amongst the four tested.

When tested for their ability to scavenge the chemical generated NO•, both *G. robertianum* extracts revealed remarkable results (20.0±0.9 and 24.2±8.0 µg/mL for GRL and GRS, respectively), indicating that, in a primary approach, these two extracts might possess strong anti-inflammatory abilities.

Since these plant extracts are intended to be used in human diet, their possible hepatotoxicity was also tested. For that, HepG-2, a human hepatocyte cell line, was cultured with different concentrations of the plant extracts (25 – 100 µg/mL and 50 – 200 µg/mL for *G. robertianum* and EAS extracts, respectively) but none revealed cytotoxic effects.

The anti-inflammatory effects of the samples were then evaluated through the inhibition of the 5-LOX activity as well as of nitrite production by RAW 264.7 macrophages. Moreover, the extract that revealed the most promising effects was also evaluated for its ability to inhibit intracellular iNOS and COX-2 expression through Western blot analysis.

Both *G. robertianum* extracts revealed remarkable effect towards 5-LOX, inhibiting approximately 65% of its activity and hence, these results suggested that the extracts could be exploited as possible therapeutic agents targeting this enzyme. GRS extract at 100 µg/mL also caused relevant inhibitory effects on nitrites production by RAW 264.7 cells. The fact that this extract did not interfered with the levels of intracellular iNOS indicates that the decreased NO• levels were resultant from the extract ability to scavenge these radicals rather than inhibiting the enzyme expression that is responsible for its production. Figure **25** resumes schematically where this extract acts during the inflammatory process.

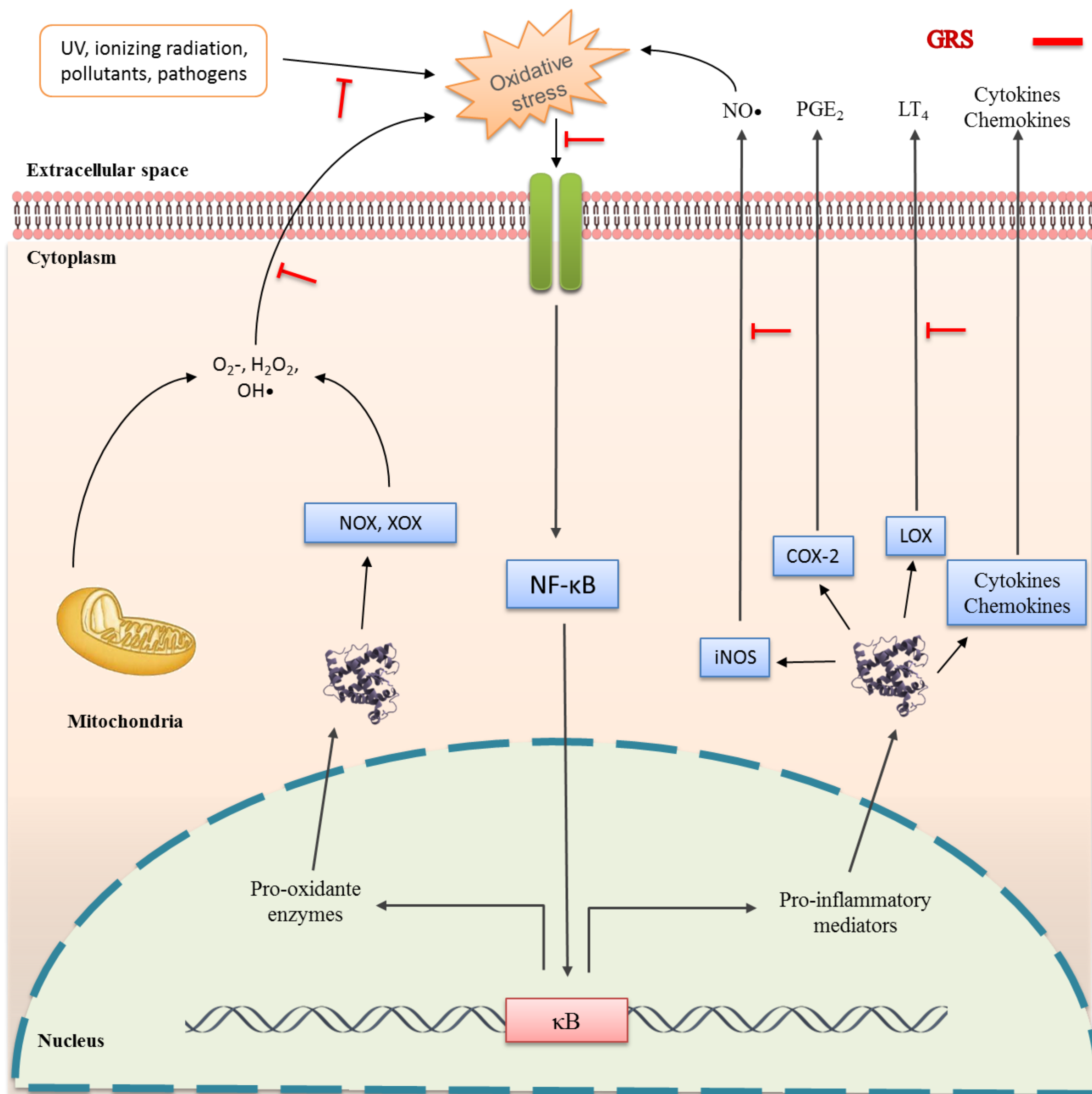


FIGURE 25 – Proposed protective mechanisms of GRS in oxidative-stress and inflammatory events. The oxidative stress stimulus trigger the activation of nuclear factor κB (NF-κB) that translocates to the nucleus where it enhances the transcription of various pro-oxidant and pro-inflammatory mediators genes including those of the enzymes nicotinamide adenine dinucleotide phosphate oxidase (NOX), xanthine oxidase (XOX), cyclooxygenase-2 (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS), as well as several cytokines and chemokines. The pro-oxidant enzymes together with mitochondrial activity will generate more reactive species that will contribute for the increase of the oxidative stress. In the presence of the GRS extract the reactive species are neutralized attenuating the oxidative stress condition. In turn, the three pro-inflammatory enzymes produce prostaglandins (PGE_2) leukotrientes (LT_4) and nitric oxide that are released in the extracellular space together with various cytokines and chemokines propagating the inflammation by transducing the signals to other cells. In presence of the GRS extract, the levels of nitric oxide produced by iNOS are reduced due to its ability to scavenge this radical. Likewise, the GRS extract interferes with the LOX activity reducing the release of leukotrienes to the extracellular space, contributing for the attenuation of the inflammatory event.

The total phenolic quantification of the four extracts demonstrated that GRL extract was the most phenolic enriched sample, while EAL extract was the poorest. Moreover, through HPLC-MS^n analysis it was observed that the *E. africanus* extracts were more abundant in chlorogenic acids while *G. robertianum* had predominance of ellagic acid and ellagitannins.

A close correlation between the antioxidant activities and the total phenolic content was noted, though there was some exceptions. E.g. in the TBARS assay the EAL extract was slightly better than the EAS extract (268.6±45.9 against 416.1±237 µg/mL, respectively) which could be partially related to its higher content in eriodictyol-hexuronide, and the EAS extract was better than the others in the ORAC assay, which might be explained not only by its abundance in chlorogenic acids but also by the presence of other non-phenolic compounds that have not been considered in this study. Moreover, the abudance of *G. robertianum* in ellagic acid might contribute to the remarkable effects of these extracts on the chemical generated $NO^\bullet$ scavenging assay. However, against what was expected, the GRL was unable to reproduce that effect on RAW 264.7 macrophages, while the GRS extract could induce a significant decrease of this radical but was ineffective in inhibiting the iNOS and COX-2 expressions. In order to confirm the *in vitro* anti-inflammatory activity of the GRS extract other pro-inflammatory mediators and signaling pathways should be addressed, namely cytokines, NF-κB transcription factor and mitogen-activated protein kinases (MAPKs).

Overall, these results demonstrate that there is some potential for these extracts to be exploited as therapeutic agents mainly due to their antioxidant properties. However, it is of utmost importance to evaluate their properties in biological models in order to better comprehend their behavior in more physiological environments. Likewise, as very promising effects were shown for *G. robertianum* in 5-LOX activity, it would be interesting to investigate in the future which compounds in the extracts better contribute for these results, as well as the type of pharmacological inhibition that is exerted over the enzyme in biological systems, i.e., if it is competitive, non-competitive or mixed inhibition. Moreover, despite *E. africanus* extracts did not show relevant effects in nitrites production by RAW 264.7, they demonstrated to somehow stimulate their metabolism. Therefore, it would be interesting to test higher concentrations of these samples for their anti-inflammatory properties, or alternatively, test their effects on other intracellular signaling cascades responsible for cell proliferation and survival such as the mitogen activated protein kinase signaling cascade.

REFERENCE LIST

7 REFERENCE LIST

ADOM, Kafui Kwami; IU, Rui Hai - Antioxidant Activity of Grains. **Journal of agricultural and food chemistry**. 50:21 (2002) 6182–6187. doi: 10.1021/jf0205099.

AMABEOKU, G. J. *et al.* - Effects of Tarchonanthus camphoratus and Eriocephalus africanus on nociception in mice and pyrexia in rats. **Phytomedicine : international journal of phytotherapy and phytopharmacology**. . ISSN 0944-7113. 7:6 (2000) 517–522. doi: 10.1016/S0944-7113(00)80038-0.

AMARAL, Sónia *et al.* - Plant extracts with anti-inflammatory properties-A new approach for characterization of their bioactive compounds and establishment of structure-antioxidant activity relationships. **Bioorganic and Medicinal Chemistry**. . ISSN 09680896. 17:5 (2009) 1876–1883. doi: 10.1016/j.bmc.2009.01.045.

ANSORENA, D. *et al.* - Development of nutraceuticals containing marine algae oils. Em DOMÍNGUEZ, HERMINIA B. T. (Ed.) - **Functional ingredients from algae for foods and nutraceuticals**. [S.l.] : Woodhead Publishing, 2013. ISBN 978-0-85709-512-1. p. 634–657.

ANTONIA ALVAREZ-FERNADEZ, M. *et al.* - Composition of Nonanthocyanin Polyphenols in Alcoholic- Fermented Strawberry Products Using LC–MS (QTRAP), High- Resolution MS (UHPLC-Orbitrap-MS), LC-DAD, and Antioxidant Activity. **Journal of agricultural and food chemistry**. 63:7 (2015) 2041–2051. doi: 10.1021/jf506076n.

AREIAS, Filipe Miguel *et al.* - Antioxidant effect of flavonoids after ascorbate/Fe2+-induced oxidative stress in cultured retinal cells. **Biochemical Pharmacology**. . ISSN 00062952. 62:1 (2001) 111–118. doi: 10.1016/S0006-2952(01)00621-9.

AYALA, Antonio; MUÑOZ, Mario F.; ARGÜELLES, Sandro - Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. **Oxidative Medicine and Cellular Longevity**. . ISSN 19420994. 2014). doi: 10.1155/2014/360438.

BALAMURUGAN, K.; KARTHIKEYAN, J. - Evaluation of luteolin in the prevention of N-nitrosodiethylamine-induced hepatocellular carcinoma using animal model system. **Indian Journal of Clinical Biochemistry**. . ISSN 09701915. 27:2 (2012) 157–163. doi: 10.1007/s12291-011-0166-7.

BASTOS, DH; SALDANHA, LA; CATHARINO, RR - Phenolic antioxidants identified by ESI-MS from yerba mate (Ilex paraguariensis) and green tea (Camelia sinensis) extracts. **Molecules**. 12:3 (2007) 423–432. doi: 10.3390/12030423.

BAYLAC, Sabine; RACINE, Philippe - Inhibition of 5-lipoxygenase by essential oils and other natural fragment extracts. **International Journal of Aromatherapy**. . ISSN 09624562. 13:2-3 (2003) 138–142. doi: 10.1016/S0962-4562(03)00083-3.

BEKIR, Jalila *et al.* - Chemical composition and antioxidant, anti-inflammatory, and antiproliferation activities of pomegranate (Punica granatum) flowers. **Journal of medicinal food**. . ISSN 1557-7600. 16:6 (2013) 544–50. doi: 10.1089/jmf.2012.0275.

BENZIE, I. F.; STRAIN, J. J. - The ferric reducing ability of plasma (FRAP) as a measure of «antioxidant power»: the FRAP assay. **Analytical biochemistry**. . ISSN 0003-2697. 239:1 (1996) 70–6. doi: 10.1006/abio.1996.0292.

BOHN, Torsten - Dietary factors affecting polyphenol bioavailability. **Nutrition Reviews**. . ISSN 17534887. 72:7 (2014) 429–452. doi: 10.1111/nure.12114.

BOR, Jung-Yi; CHEN, Hui-Yin; YEN, Gow-Chin - Evaluation of antioxidant activity and inhibitory effect on nitric oxide production of some common vegetables. **Journal of agricultural and food chemistry**. . ISSN 0021-8561. 54:5 (2006) 1680–1686. doi: 10.1021/jf0527448.

BUFALO, M. C. *et al.* - Propolis and its constituent caffeic acid suppress LPS-stimulated pro-inflammatory response by blocking NF-κB and MAPK activation in macrophages. **Journal of Ethnopharmacology**. . ISSN 0378-8741. 149:1 (2013) 84–92. doi: 10.1016/j.jep.2013.06.004.

CAI, Yi-zhong *et al.* - Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. **Life sciences**. . ISSN 00243205. 78:25 (2006) 2872–2888. doi: 10.1016/j.lfs.2005.11.004.

CARDONA, Fernando *et al.* - Benefits of polyphenols on gut microbiota and implications in human health. **The Journal of nutritional biochemistry**. . ISSN 1873-4847. 24:8 (2013) 1415–22. doi: 10.1016/j.jnutbio.2013.05.001.

CARDOSO, Susana M. *et al.* - Virgin olive oil as a source of anti-inflammatory agents. Em LEONARDIS, ANTONELLA DE (Ed.) - **Virgin Olive Oil: Production, Composition, Uses and Benefits for Man**. New York : Nova Science Publishers, 2014

CARREAU, Charlotte *et al.* - Enterodiol and enterolactone, two major diet-derived polyphenol metabolites have different impact on ERα transcriptional activation in human breast cancer cells. **Journal of Steroid Biochemistry and Molecular Biology**. . ISSN 09600760. 110:1-2 (2008) 176–185. doi: 10.1016/j.jsbmb.2008.03.032.

CATARINO, Marcelo D. *et al.* - Characterization of phenolic constituents and evaluation of antioxidant properties of leaves and stems of Eriocephalus africanus. **Arabian Journal of Chemistry**. . ISSN 18785352. 2015). doi: 10.1016/j.arabjc.2015.04.018.

CATARINO, Marcelo D. *et al.* - Antioxidant Capacities of Flavones and Benefits in Oxidative-Stress Related Diseases. **Current Topics in Medicinal Chemistry**. . ISSN 1873-4294 (Electronic). 15:2 (2015) 105–119.

CATARINO, Marcelo Dias *et al.* - Mediaterranean Diet: A Precious Tool for Fighting Inflammatory Diseases. Em COBB, DEAN T. (Ed.) - **Polyphenols: Food Sources, Bioactive Properties and Antioxidant Effects**. New York : Nova Science Publishers, 2014. ISBN 978-1-63117-858-0. p. 87–112.

CHANDRASEKARA, Anoma; SHAHIDI, Fereidoon - Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. **Journal of Agricultural and Food Chemistry**. . ISSN 00218561. 58:11 (2010) 6706–6714. doi: 10.1021/jf100868b.

CHANG, Yuan-Ching *et al.* - Structure-activity relationship of C6-C3 phenylpropanoids on xanthine oxidase-inhibiting and free radical-scavenging activities. **Biochemical Pharmacology**. . ISSN 00062952. 43:11 (2007) 1541 – 1551. doi: 10.1016/j.bcp.2007.11.023.

CHIANG, Yi-ming *et al.* - Ethyl caffeate suppresses NF-kappaB activation and its downstream inflammatory mediators, iNOS, COX-2, and PGE2 in vitro or in mouse skin. **British journal of pharmacology**. . ISSN 0007-1188. 146:3 (2005) 352–363. doi: 10.1038/sj.bjp.0706343.

CILLA, Antonio *et al.* - Foods or Bioactive Constituents of Foods as Chemopreventives in Cell Lines After Simulated Gastrointestinal Digestion: A Review. Em MORALES-GONZÁLEZ, JOSÉ A. (Ed.) - **Oxidative Stress and Chronic Degenerative Diseases - A Role for Antioxidants**. ISBN 978-953-51-1123-8. p. 131–151.

CLIFFORD, Michael N. *et al.* - Hierarchical scheme for LC-MSn identification of chlorogenic acids. **Journal of Agricultural and Food Chemistry**. . ISSN 00218561. 51:1 (2003) 2900–2911. doi: 10.1021/jf026187q.

CLIFFORD, Michael N.; KNIGHT, Susan; KUHNERT, Nikolai - Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS(n). **Journal of agricultural and food chemistry**. . ISSN 0021-8561. 53:10 (2005) 3821–3832. doi: 10.1021/jf050046h.

COMALADA, M.; XAUS, J.; GÁLVEZ, J. - **Bioactive Food as Dietary Interventions for Arthritis and Related Inflammatory Diseases**. [S.l.] : Elsevier, 2013. ISBN 9780123971562.

COMALADA, Mònica *et al.* - Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship. **Biochemical pharmacology**. . ISSN 0006-2952. 72:8 (2006) 1010–21. doi: 10.1016/j.bcp.2006.07.016.

COS, Paul *et al.* - Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. **Journal of Natural Products**. . ISSN 01633864. 61:1 (1998) 71–76. doi: 10.1021/np970237h.

CRAGG, Gordon M.; NEWMAN, David J. - Natural products: a continuing source of novel drug leads. **Biochimica et biophysica acta**. . ISSN 0006-3002. 1830:6 (2013) 3670–95. doi: 10.1016/j.bbagen.2013.02.008.

CROZIER, Alan; RIO, Daniele DEL; CLIFFORD, Michael N. - Bioavailability of dietary flavonoids and phenolic compounds. **Molecular aspects of medicine**. . ISSN 1872-9452. 31:6 (2010) 446–67. doi: 10.1016/j.mam.2010.09.007.

D'ARCHIVIO, Massimo *et al.* - Polyphenols, dietary sources and bioavailability. **Annali dell'Istituto Superiore di Sanita**. . ISSN 00212571. 43:4 (2007) 348–361. doi: Vol. 43, no. 4: 348-361.

D'ARCHIVIO, Massimo *et al.* - Bioavailability of the polyphenols: Status and controversies. **International Journal of Molecular Sciences**. . ISSN 14220067. 11:4 (2010) 1321–1342. doi: 10.3390/ijms11041321.

DAGLIA, Maria - Polyphenols as antimicrobial agents. **Current Opinion in Biotechnology**. . ISSN 09581669. 23:2 (2012) 174–181. doi: 10.1016/j.copbio.2011.08.007.

DIXON, Richard A; PASINETTI, Giulio M. - Flavonoids and isoflavonoids: from plant biology to agriculture and neuroscience. **Plant physiology**. . ISSN 1532-2548. 154:2 (2010) 453–7. doi: 10.1104/pp.110.161430.

DRÖGE, Wulf - Free radicals in the physiological control of cell function. **Physiological reviews**. . ISSN 0031-9333. 82:1 (2002) 47–95. doi: 10.1152/physrev.00018.2001.

ERMER, J.; MILLER, J. M.; ERMER JOACHIM, John Miller - **Method Validation in Pharmaceutical Analysis: A Guide to Best Practice**. Weinheim : Wiley-VCH Verlag GmbH & Co., 2005

ESPÍN, Juan Carlos *et al.* - Biological Significance of Urolithins, the Gut Microbial Ellagic Acid-Derived Metabolites: The Evidence So Far. **Evidence-based complementary and alternative medicine**. New York. 2013). doi: 10.1155/2013/270418.

ESPÍN, Juan Carlos; GARCÍA-CONESA, María Teresa; TOMÁS-BARBERÁN, Francisco A. - Nutraceuticals: Facts and fiction. **Phytochemistry**. . ISSN 00319422. 68:22-24 (2007) 2986–3008. doi: 10.1016/j.phytochem.2007.09.014.

FEDERICO, Alessandro *et al.* - Chronic inflammation and oxidative stress in human carcinogenesis. **International Journal of Cancer**. . ISSN 00207136. 121:11 (2007) 2381–2386. doi: 10.1002/ijc.23192.

FERNANDES, Iva *et al.* - Bioavailability of anthocyanins and derivatives. **Journal of Functional Foods**. . ISSN 17564646. 7:1 (2014) 54–66. doi: 10.1016/j.jff.2013.05.010.

FERREIRA, Fernanda M. *et al.* - «MitoTea»: Geranium robertianum L. decoctions decrease blood glucose levels and improve liver mitochondrial oxidative phosphorylation in diabetic Goto-Kakizaki rats. **Acta Biochimica Polonica**. 57:4 (2010) 399–402.

FERREYRA, María L. Falcone; RIUS, Sebastián P.; CASATI, Paula - Flavonoids: biosynthesis, biological functions, and biotechnological applications. **Frontiers in Plant Science**. 3:222 (2012) 1–15. doi: 10.3389/fpls.2012.00222.

FISCHER, Ulrike A.; CARLE, Reinhold; KAMMERER, Dietmar R. - Identification and quantification of phenolic compounds from pomegranate (Punica granatum L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD-ESI/MSn. **Food Chemistry**. . ISSN 03088146. 127:2 (2011) 807–821. doi: 10.1016/j.foodchem.2010.12.156.

FODOREA, Cristina-Stefania *et al.* - Preliminary HPLC study on some polyphenols of Geranium robertianum L. (Geraniaceae). **Revista medico-chirurgicala a Societatii de Medici si Naturalisti din Iasi**. Romania. . ISSN 0048-7848. 109:1 (2005) 174–178.

FRACASSETTI, Daniela *et al.* - Ellagic acid derivatives, ellagitannins, proanthocyanidins and other phenolics, vitamin C and antioxidant capacity of two powder products from camu-camu fruit (Myrciaria dubia). **Food Chemistry**. . ISSN 03088146. 139:1-4 (2013) 578–588. doi: 10.1016/j.foodchem.2013.01.121.

FREITAS, Pedro Manuel Marques DE - **Polyphenols and Neurodegenerative Diseases**. [S.l.] : University of Porto, 2012

GARAZD, M. M.; GARAZD, Ya. L.; KHILYA, V. P. - Neoflavones. 1. Natural Distribution and Spectral and Biological Properties. **Chemistry of natural compounds**. 39:1 (2003) 47–82.

GERTZ, Melanie *et al.* - A Molecular Mechanism for Direct Sirtuin Activation by Resveratrol. **PLoS ONE**. . ISSN 19326203. 7:11 (2012). doi: 10.1371/journal.pone.0049761.

GHARRAS, Hasna El - Polyphenols: Food sources, properties and applications - A review. **International Journal of Food Science and Technology**. . ISSN 09505423. 44:12 (2009) 2512–2518. doi: 10.1111/j.1365-2621.2009.02077.x.

GÓMEZ-LECHÓN, M. J. *et al.* - In vitro evaluation of potential hepatotoxicity induced by drugs. **Current pharmaceutical design**. . ISSN 13816128. 16:17 (2010) 1963–1977. doi: 10.2174/138161210791208910.

GONZÁLEZ, R. *et al.* - Effects of flavonoids and other polyphenols on inflammation. **Critical reviews in food science and nutrition**. . ISSN 1549-7852. 51:4 (2011) 331–62. doi: 10.1080/10408390903584094.

GU, JK; ZHONG, DF; CHEN, XY - Analysis of O-glucuronide conjugates in urine by electrospray ion trap mass spectrometry. **Fresenius' journal of analytical chemistry**. 365:6 (1999) 553–558. doi: 10.1007/s002160051521.

GU, Liwei *et al.* - Concentrations of proanthocyanidins in common foods and estimations of normal consumption. **The Journal of nutrition**. . ISSN 0022-3166. 134:3 (2004) 613–617.

HAGERMAN, Ann E. *et al.* - High Molecular Weight Plant Polyphenolics (Tannins) as Biological Antioxidants. **Journal of Agricultural and Food Chemistry**. 46:5 (1998) 1887–1892. doi: 10.1021/jf970975b.

HE, Zhiyong; XIA, Wenshui - Analysis of phenolic compounds in Chinese olive (Canarium album L.) fruit by RPHPLC-DAD-ESI-MS. **Food Chemistry**. . ISSN 03088146. 105:3 (2007) 1307–1311. doi: 10.1016/j.foodchem.2007.04.049.

HEIM, Kelly E.; TAGLIAFERRO, Anthony R.; BOBILYA, Dennis J. - Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. **The Journal of Nutritional Biochemistry**. . ISSN 09552863. 13:10 (2002) 572–584. doi: 10.1016/S0955-2863(02)00208-5.

HIGDON, Jane V; FREI, Balz - Tea Catechins and Polyphenols: Health Effects, Metabolism, and Antioxidant Functions. **Critical Reviews in Food Science and Nutrition**. 43:1 (2003) 89–143. doi: 10.1080/10408690390826464.

HOOPER, Lee *et al.* - Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. **The American journal of clinical nutrition**. United States. . ISSN 1938-3207. 88:1 (2008) 38–50.

ISHIMOTO, Hidekazu *et al.* - Antioxidative Properties of Functional Polyphenols and Their Metabolites Assessed by an ORAC Assay. **Bioscience, Biotechnology, and Biochemistry**. . ISSN 0916-8451. 76:2 (2012) 395–399. doi: 10.1271/bbb.110717.

IWASHINA, T. - The structure and distribution of the flavonoids in plants. **Journal of Plant Research**. 113:3 (2000) 287–299. doi: 10.1007/PL00013940.

JEMIA, Mariem Ben *et al.* - Antioxidant activity of Tunisian Geranium robertianum L. (Geraniaceae). **Natural product research**. . ISSN 1478-6427. 27:22 (2013) 2076–83. doi: 10.1080/14786419.2013.782492.

JURD, Leonard - **Recent progress in the chemistry of flavylium salts**. [S.l.] : Elsevier, 2013. ISBN 9781483220017.

KAYANO, Shin Ichi *et al.* - Antioxidant activity of prune (Prunus domestica L.) constituents and a new synergist. **Journal of Agricultural and Food Chemistry**. . ISSN 00218561. 50:13 (2002) 3708–3712. doi: 10.1021/jf0200164.

KHANSARI, Nemat; SHAKIBA, Yadollah; MAHMOUDI, Mahdi - Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. **Recent patents on inflammation & allergy drug discovery**. 3:1 (2009) 73–80. doi: 10.2174/187221309787158371.

KIM, Hyun Pyo *et al.* - Anti-inflammatory plant flavonoids and cellular action mechanisms. **Journal of pharmacological sciences**. . ISSN 1347-8613. 96:3 (2004) 229–245. doi: 10.1254/jphs.CRJ04003X.

KIM, Kyung Hee *et al.* - Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. **Food Chemistry**. . ISSN 03088146. 95:3 (2006) 466–473. doi: 10.1016/j.foodchem.2005.01.032.

KNAZE, Viktoria *et al.* - Intake estimation of total and individual flavan-3-ols, proanthocyanidins and theaflavins, their food sources and determinants in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. **British Journal of Nutrition**. . ISSN 0007-1145. 108:06 (2012) 1095–1108. doi: 10.1017/S0007114511006386.

KOBAKHIDZE, K. B.; ALANIYA, M. D. - Flavonoids from Geranium robertianum. **CHEMISTRY OF NATURAL COMPOUNDS**. . ISSN 0009-3130. 40:1 (2004) 89–90. doi: 10.1023/B:CONC.0000025477.18086.8b.

KUMAR, Ramanathan Sambath *et al.* - Antimicrobial and antioxidant activities of Careya arborea Roxb. stem bark. **Iranian Journal of Pharmacology and Therapeutics**. . ISSN 17352657. 5:1 (2006) 35–41.

KUMAR, Shashank; PANDEY, Abhay K. - Chemistry and biological activities of flavonoids: an overview. **The Scientific World Journal**. 2013). doi: 10.1155/2013/162750.

LAMPORT, Daniel J. *et al.* - The effects of flavonoid and other polyphenol consumption on cognitive performance : A systematic research review of human experimental and epidemiological studies. **Nutrition and Aging**. 1:2012) 5–25. doi: 10.3233/NUA-2012-0002.

LANDETE, J. M. - Updated knowledge about polyphenols: functions, bioavailability, metabolism, and health. **Critical reviews in food science and nutrition**. . ISSN 1549-7852. 52:10 (2012) 936–48. doi: 10.1080/10408398.2010.513779.

LARROSA, Mar *et al.* - Ellagitannins, ellagic acid and vascular health. **Molecular aspects of medicine**. . ISSN 1872-9452. 31:6 (2010) 513–39. doi: 10.1016/j.mam.2010.09.005.

LEE, Eun Ju *et al.* - Phenolic constituents from the flower buds of Lonicera japonica and their 5-lipoxygenase inhibitory activities. **Food Chemistry**. . ISSN 03088146. 120:1 (2010) 134–139. doi: 10.1016/j.foodchem.2009.09.088.

LEE, Joon Hee; JOHNSON, Jodie V.; TALCOTT, Stephen T. - Identification of ellagic acid conjugates and other polyphenolics in muscadine grapes by HPLC-ESI-MS. **Journal of Agricultural and Food Chemistry**. . ISSN 00218561. 53:15 (2005) 6003–6010. doi: 10.1021/jf050468r.

LEOPOLDINI, Monica *et al.* - Antioxidant properties of phenolic compounds: H-atom versus electron transfer mechanism. **Journal of Physical Chemistry A**. . ISSN 10895639. 108:22 (2004) 4916–4922. doi: 10.1021/jp037247d.

LIU, Rui Hai - Potential synergy of phytochemicals in cancer prevention: mechanism of action. **The Journal of nutrition**. . ISSN 0022-3166. 134:12 Suppl (2004) 3479S–3485S. doi: 134/12/3479S [pii].

MADE, Sanne M. VAN DER; MENSINK, Ronald P. - Polyphenols for Cholesterol Management. Em GARG, ABHIMANYU (Ed.) - **Dyslipidemias SE - 22**. [S.l.] : Humana Press, 2015. ISBN 978-1-60761-423-4. p. 371–382.

MANACH, Claudine *et al.* - Polyphenols: food sources and bioavailability. **The American journal of clinical nutrition**. . ISSN 0002-9165. 79:5 (2004) 727–747.

MANACH, Claudine *et al.* - Bioavailability and bioefficacy of polyphenols in humans. I. Review of 93 intervention studies. **American Journal of Clinical Nutrition**. . ISSN 00029165. 81:1 Suppl (2005) 243S–255S. doi: 81/1/230S [pii].

MANSOURI, Mohammad Taghi *et al.* - A study of the mechanisms underlying the anti-inflammatory effect of ellagic acid in carrageenan-induced paw edema in rats. **Indian Journal of Pharmacology**. India. . ISSN 0253-7613. 47:3 (2015) 292–298. doi: 10.4103/0253-7613.157127.

MAQSOOD, Sajid; BENJAKUL, Soottawat - Comparative studies of four different phenolic compounds on in vitro antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince. **Food Chemistry**. . ISSN 03088146. 119:1 (2010) 123–132. doi: 10.1016/j.foodchem.2009.06.004.

MARAIS, Jannie P. J. *et al.* - The Stereochemistry of Flavonoids. Em GROTEWOLD, ERICH (Ed.) - **The Science of Flavonoids**. Ohio : Springer Science+Business Media, Inc., 2006. p. 1–46.

MCCUNE, Letitia M. *et al.* - Cherries and health: a review. **Critical reviews in food science and nutrition**. . ISSN 1040-8398. 51:1 (2011) 1–12. doi: 10.1080/10408390903001719.

MEIRELES, Gyselle Cynthia Silva; SANTOS, Adrielle Mangabeira - Mecanismos de ação da laserterapia sobre componentes do processo inflamatório. **Revista Eletrônica da Fainor**. 3:1 (2010) 30–40.

MENA, Pedro *et al.* - Rapid and comprehensive evaluation of (Poly)phenolic compounds in pomegranate (Punica granatum L.) Juice by UHPLC-MSn. **Molecules**. . ISSN 14203049. 17:12 (2012) 14821–14840. doi: 10.3390/molecules171214821.

MERLE, Hugo *et al.* - Chemical composition of the essential oils from Eriocephalus africanus L. var. africanus populations growing in Spain. **Flavour and Fragrance Journal**. . ISSN 0882-5734. 22:6 (2007) 461–464.

MEYERS, Katherine J.; SWIECKI, Tedmund J.; MITCHELL, Alyson E. - Understanding the Native Californian diet: Identification of condensed and hydrolyzable tannins in tanoak acorns (Lithocarpus densiflorus). **Journal of Agricultural and Food Chemistry**. . ISSN 00218561. 54:20 (2006) 7686–7691. doi: 10.1021/jf061264t.

MULLEN, W. *et al.* - The antioxidant and chlorogenic acid profiles of whole coffee fruits are influenced by the extraction procedures. **Journal of Agricultural and Food Chemistry**. . ISSN 00218561. 59:8 (2011) 3754–3762. doi: 10.1021/jf200122m.

MULLEN, William *et al.* - Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC-MSn. **Phytochemistry**. . ISSN 00319422. 64:2 (2003) 617–624. doi: 10.1016/S0031-9422(03)00281-4.

NEAGU, Elena *et al.* - Evaluation of antioxidant capacity of Geranium robertianum extracts. **Revue Roumaine de Chimie**. . ISSN 00353930. 55:6 (2010) 321–325.

NJENGA, E. W.; VILJOEN, A. M. - In vitro 5-lipoxygenase inhibition and anti-oxidant activity of Eriocephalus L. (Asteraceae) species. **South African Journal of Botany**. . ISSN 02546299. 72:4 (2006) 637–641.

NJENGA, Elizabeth Wanjiku - **The chemotaxonomy, Phylogeny and Biological Activity of the Genus Eriocephalus L. (Asteraceae)**. [S.l.] : Faculty of Health Sciences, University of the Witwatersrand, 2005

NORTH AMERICAN MENOPAUSE SOCIETY - The role of soy isoflavones in menopausal health: report of The North American Menopause Society/Wulf H. Utian Translational Science Symposium in Chicago, IL (October 2010). **Menopause**. United States. . ISSN 1530-0374. 18:7 (2011) 732–753. doi: 10.1097/gme.0b013e31821fc8e0.

PANDEY, Kanti Bhooshan; RIZVI, Syed Ibrahim - Plant polyphenols as dietary antioxidants in human health and disease. **Oxidative medicine and cellular longevity**. . ISSN 1942-0900. 2:5 (2009) 270–278. doi: 10.4161/oxim.2.5.9498.

PARK, Jae B. - Isolation and quantification of major chlorogenic acids in three major instant coffee brands and their potential effects on H2O2-induced mitochondrial membrane depolarization and apoptosis in PC-12 cells. **Food & function**. England. 4:11 (2013) 1632–1638. doi: 10.1039/c3fo60138b.

PAUN, Gabriela *et al.* - Evaluation of Geranium spp., Helleborus spp. and Hyssopus spp. polyphenolic extracts inhibitory activity against urease and α-chymotrypsin. **Journal of enzyme inhibition and medicinal chemistry**. . ISSN 1475-6374. 6366:1 (2013) 1–7. doi: 10.3109/14756366.2012.749399.

PEREIRA, David M. *et al.* - Phenolics: From chemistry to biology. **Molecules**. . ISSN 14203049. 14:6 (2009) 2202–2211. doi: 10.3390/molecules14062202.

PEREIRA, Olívia R. *et al.* - Phenolic constituents of Lamium album: Focus on isoscutellarein derivatives. **Food Research International**. . ISSN 09639969. 48:1 (2012) 330–335. doi: 10.1016/j.foodres.2012.04.009.

PEREIRA, Olívia R. *et al.* - Simultaneous characterization and quantification of phenolic compounds in Thymus x citriodorus using a validated HPLC–UV and ESI–MS combined method. **Food Research International**. . ISSN 09639969. 54:2 (2013) 1773–1780.

PERRON, Nathan R.; BRUMAGHIM, Julia L. - A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. **Cell Biochemistry and Biophysics**. . ISSN 10859195. 53:2 (2009) 75–100. doi: 10.1007/s12013-009-9043-x.

PETRUSSA, Elisa *et al.* - Plant flavonoids-biosynthesis, transport and involvement in stress responses. **International Journal of Molecular Sciences**. . ISSN 16616596. 14:7 (2013) 14950–14973. doi: 10.3390/ijms140714950.

PIWOWARSKI, Jakub P. *et al.* - Role of human gut microbiota metabolism in the anti-inflammatory effect of traditionally used ellagitannin-rich plant materials. **Journal of Ethnopharmacology**. . ISSN 18727573. 155:1 (2014) 801–809. doi: 10.1016/j.jep.2014.06.032.

PORRINI, Marisa; RISO, Patrizia - Factors influencing the bioavailability of antioxidants in foods: A critical appraisal. **Nutrition, Metabolism and Cardiovascular Diseases**. . ISSN 09394753. 18:10 (2008) 647–650. doi: 10.1016/j.numecd.2008.08.004.

PUUPPONEN-PIMIÄ, R. *et al.* - Antimicrobial properties of phenolic compounds from berries. **Journal of Applied Microbiology**. . ISSN 13645072. 90:4 (2001) 494–507. doi: 10.1046/j.1365-2672.2001.01271.x.

RASHIDINEJAD, Ali *et al.* - Effects of catechin on the phenolic content and antioxidant properties of low-fat cheese. **International Journal of Food Science and Technology**. . ISSN 09505423. 48:12 (2013) 2448–2455. doi: 10.1111/ijfs.12234.

REUTER, Simone *et al.* - Oxidative stress, inflammation, and cancer: How are they linked? **Free Radical Biology and Medicine**. 49:11 (2010) 1603–1616. doi: 10.1016/j.freeradbiomed.2010.09.006.Oxidative.

RIBEIRO, Daniela *et al.* - Inhibition of LOX by flavonoids: A structure-activity relationship study. **European Journal of Medicinal Chemistry**. . ISSN 02235234. 72:2014) 137–145. doi: 10.1016/j.ejmech.2013.11.030.

RIBEIRO, Daniela *et al.* - Inhibition of LOX by flavonoids: a structure-activity relationship study. **European journal of medicinal chemistry**. . ISSN 1768-3254. 72:2014) 137–45. doi: 10.1016/j.ejmech.2013.11.030.

RICHARD-FORGET, Florence *et al.* - Inhibition of Horse Bean and Germinated Barley Lipoxygenases by Some Phenolic Compounds. **Journal of Food Science**. . ISSN 1750-3841. 60:6 (1995) 1325–1329. doi: 10.1111/j.1365-2621.1995.tb04583.x.

RODRIGUES, Naira Poerner; TOLEDO BENASSI, Marta; BRAGAGNOLO, Neura - Scavenging capacity of coffee brews against oxygen and nitrogen reactive species and the correlation with bioactive compounds by multivariate analysis. **Food Research International**. . ISSN 09639969. 61:2014) 228–235. doi: 10.1016/j.foodres.2013.09.028.

ROMAN, G. Paun *et al.* - Antioxidant activity of Geranium robertianum concentrated extracts by ultrafiltration process. **Planta Medica**. . ISSN 0032-0943. 75:09 (2009) 4–5. doi: 10.1055/s-0029-1234557.

SALIE, F.; EAGLES, P. F.; LENG, H. M. - Preliminary antimicrobial screening of four South African Asteraceae species. **Journal of ethnopharmacology**. . ISSN 0378-8741. 52:1 (1996) 27–33.

SANDHAR, Harleen Kaur *et al.* - A review of phytochemistry and pharmacology of flavonoids. **Internationale Pharmaceutica Sciencia**. 1:1 (2011) 25–41.

SANTANGELO, Carmela *et al.* - Polyphenols, intracellular signalling and inflammation. **Annali dell'Istituto superiore di sanità**. . ISSN 0021-2571. 43:4 (2007) 394–405.

SANTHAKUMAR, A. B.; BULMER, A. C.; SINGH, I. - A review of the mechanisms and effectiveness of dietary polyphenols in reducing oxidative stress and thrombotic risk. **Journal of Human Nutrition and Dietetics**. . ISSN 09523871. 27:1 (2014) 1–21. doi: 10.1111/jhn.12177.

SCALBERT, Augustin; WILLIAMSON, Gary - Dietary Intake and Bioavailability of Polyphenols. **Journal of Medicinal Food**. . ISSN 1096-620X. 3:2 (2000) 121–125. doi: 10.1089/109662000416311.

SCHÜTZ, Katrin *et al.* - Identification and quantification of caffeoylquinic acids and flavonoids from artichoke (Cynara scolymus L.) heads, juice, and pomace by HPLC-DAD-ESI/MS(n). **Journal of agricultural and food chemistry**. . ISSN 0021-8561. 52:13 (2004) 4090–4096.

SEERAM, Navindra P. - Polyphenols Overview. Em **Encyclopedia of Dietary Supplements, Second Edition**. [S.l.] : CRC Press, 2010. ISBN 978-1-4398-1928-9. p. 632–634.

SHARMA, J. N.; AL-OMRAN, A.; PARVATHY, S. S. - Role of nitric oxide in inflammatory diseases. **Inflammopharmacology**. Switzerland. . ISSN 0925-4692 (Print). 15:6 (2007) 252–259. doi: 10.1007/s10787-007-0013-x.

SILK, Jennifer J.; SMOLIGA, James M. - Resveratrol: Nutraceutical believed to counteract the detrimental effects of high-fat diet. **Lipid Technology**. . ISSN 18635377. 26:1 (2014) 15–17. doi: 10.1002/lite.201300315.

SLIMESTAD, Rune; FOSSEN, Torgils; VÅGEN, Ingunn Molund - Onions: A source of unique dietary flavonoids. **Journal of Agricultural and Food Chemistry**. . ISSN 00218561. 55:25 (2007) 10067–10080. doi: 10.1021/jf0712503.

SMITH, P. K. *et al.* - Measurement of protein using bicinchoninic acid. **Analytical Biochemistry**. . ISSN 00032697. 150:1 (1985) 76–85. doi: 10.1016/0003-2697(85)90442-7.

SNYDER, L. R.; KIRKLAND, J. J.; DOLAN, J. W. - **Introduction to modern liquid chromatography**. 3th. ed. New Jersey : John Wiley & Sons, Inc., 2009. ISBN 0471038229.

SRIVASTAVA, Anup; JAGAN MOHAN RAO, L.; SHIVANANDAPPA, T. - Isolation of ellagic acid from the aqueous extract of the roots of Decalepis hamiltonii: Antioxidant activity and cytoprotective effect. **Food Chemistry**. . ISSN 03088146. 103:1 (2007) 224–233. doi: 10.1016/j.foodchem.2006.08.010.

SVENDSEN, Mathias T. *et al.* - Contact sensitization to *Geranium robertianum* L. in an amateur gardener. **Contact Dermatitis**. . ISSN 01051873. 72:6 (2015) 420–421. doi: 10.1111/cod.12378.

TAKANO-ISHIKAWA, Y.; GOTO, M.; YAMAKI, K. - Structure-activity relations of inhibitory effects of various flavonoids on lipopolysaccharide-induced prostaglandin E2 production in rat peritoneal macrophages: comparison between subclasses of flavonoids. **Phytomedicine : international journal of phytotherapy and phytopharmacology**. . ISSN 0944-7113. 13:5 (2006) 310–7. doi: 10.1016/j.phymed.2005.01.016.

TANWAR, Beenu; MODGIL, Rajni - Flavonoids: Dietary Occurrence and Health Benefits. **Spatula DD - Peer Reviewed Journal on Complementary Medicine and Drug Discovery**. . ISSN 1309-9914. 2:1 (2012) 59. doi: 10.5455/spatula.20120328100506.

TAPPEL, A L.; LUNDBERG, W. O.; BOYER, P. D. - Effect of temperature and antioxidants upon the lipoxidase-catalyzed oxidation of sodium linoleate. **Archives of biochemistry and biophysics**. . ISSN 00039861. 42:2 (1953) 293–304. doi: 10.1016/0003-9861(53)90359-2.

THATOI, H. N.; PATRA, J. K.; DAS, S. K. - Free radical scavenging and antioxidant potential of mangrove plants: a review. **Acta Physiologiae Plantarum**. . ISSN 0137-5881. 36:3 (2013) 561–579. doi: 10.1007/s11738-013-1438-z.

TOFTS, Richard J. - Geranium robertianum L. **Journal of Ecology**. . ISSN 00220477. 92:3 (2004) 537–555. doi: 10.1111/j.0022-0477.2004.00892.x.

TOMÁS-BARBERÁN, Francisco A.; CLIFFORD, Michael N. - Flavanones, chalcones and dihydrochalcones - Nature, occurrence and dietary burden. **Journal of the Science of Food and Agriculture**. . ISSN 00225142. 80:7 (2000) 1024–1032. doi: 10.1002/(SICI)1097-0010(20000515)80:7<1024::AID-JSFA567>3.0.CO;2-S.

TSAO, Rong - Chemistry and biochemistry of dietary polyphenols. **Nutrients**. . ISSN 2072-6643. 2:12 (2010) 1231–46. doi: 10.3390/nu2121231.

UMESALMA, Syed; SUDHANDIRAN, Ganapasam - Differential inhibitory effects of the polyphenol ellagic acid on inflammatory mediators NF-κB, iNOS, COX-2, TNF-α, and IL-6 in 1,2-dimethylhydrazine-induced rat colon carcinogenesis. **Basic and Clinical Pharmacology and Toxicology**. . ISSN 17427835. 107:2 (2010) 650–655. doi: 10.1111/j.1742-7843.2010.00565.x.

VARDENY, Orly; SOLOMON, Scott D. - Cyclooxygenase-2 inhibitors, nonsteroidal anti-inflammatory drugs, and cardiovascular risk. **Cardiology clinics**. . ISSN 1558-2264. 26:4 (2008) 589–601. doi: 10.1016/j.ccl.2008.06.004.

VEBERIC, Robert *et al.* - Anthocyanin composition of different wild and cultivated berry species. **LWT - Food Science and Technology**. . ISSN 00236438. 60:1 (2015) 509–517. doi: 10.1016/j.lwt.2014.08.033.

WEISZ, Georg M.; KAMMERER, Dietmar R.; CARLE, Reinhold - Identification and quantification of phenolic compounds from sunflower (Helianthus annuus L.) kernels and shells by HPLC-DAD/ESI-MSn. **Food Chemistry**. . ISSN 03088146. 115:2 (2009) 758–765.

XIAO, Jianbo; MUZASHVILI, Tamar S.; GEORGIEV, Milen I. - Advances in the biotechnological glycosylation of valuable flavonoids. **Biotechnology advances**. . ISSN 1873-1899. 32:6 (2014) 1145–56. doi: 10.1016/j.biotechadv.2014.04.006.

YANG, Yong-Jin *et al.* - Radical scavenging activity and cytotoxicity of active quinic acid derivatives from Scorzonera divaricata roots. **Food chemistry**. . ISSN 0308-8146. 138:2-3 (2013) 2057–63.

YEN, Gow Chin; DUH, Pin Der - Scavenging Effect of Methanolic Extracts of Peanut Hulls on Free-Radical and Active-Oxygen Species. **Journal of Agricultural and Food Chemistry**. 42:3 (1994) 629–632.

ZHANG, Xu *et al.* - Chlorogenic acid protects mice against lipopolysaccharide-induced acute lung injury. **Injury**. . ISSN 00201383. 41:7 (2010) 746–752. doi: 10.1016/j.injury.2010.02.029.